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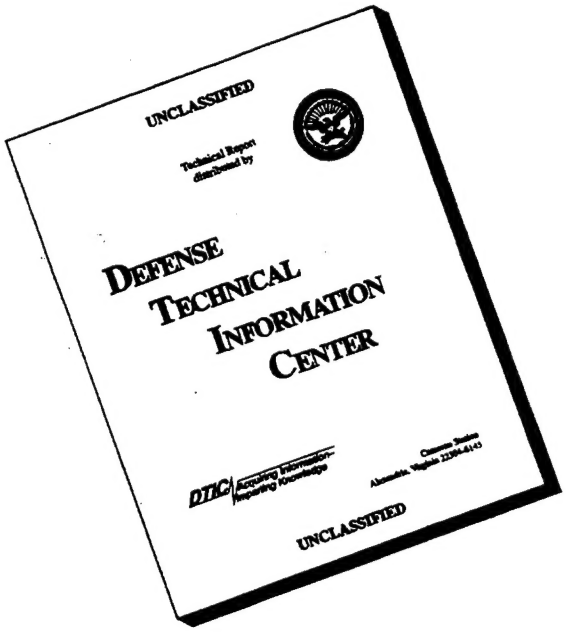

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Multiple approaches employing the tools of biochemistry, immunology, molecular biology, microscopy, and pharmacology have been used to gain greater insight into phospholipase A_2 (PLA_2) structure, function, and solution properties. Protein sequencing has enabled us to identify suspected residues and molecular domains of PLA_2s, expressing neurotoxicity, myotoxicity, hemorrhage, hypotension, and dimerization; and DNA sequencing of both cDNAs and genomic DNA has provided us with a better understanding of genomic structure, possible regulatory elements within these genes, and insights into PLA_2 gene evolution. Chemical modifications have suggested whether alterations may be involved in modifying PLA_2 function *in vivo*, and whether subunit dissociation is essential for function. NMR and x-ray diffraction have been initiated and should eventually allow predictions about other 'critical' residues. We expressed both subunits of Mojave toxin in *E. coli*, and procedures need to be developed to recover these products in biologically active forms. Once accomplished, site-specific mutagenesis studies coupled with expression to confirm that certain residues are involved in specific functions can be conducted. Pharmacological experiments have suggested that PLA_2 neurotoxins do not appear to be endocytosed to exert their effects, unlike most potent enzymatic toxins. Endogenous sera and venom toxin inhibitors have been identified and partially characterized.

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Ivan J. Kain 6-24-96

PI - Signature Date

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INTRODUCTION
Nature of the Problem

Recent reports in the literature suggested that certain covalent modifications of non-toxic phospholipases A_2 (PLA_2), significantly enhanced their phospholipase activity. We wanted to examine presynaptic neurotoxins for possible covalent modifications (acylation, transglutamination) that might potentiate neurotoxin activity *in vivo*. If such modification were found to enhance neurotoxicity, new therapy approaches would be suggested.

Glycoprotein analysis, cross-linking/cleavage, NMR, and alkylation studies are structural investigations designed to give a better understanding of neurotoxin structure and function. The proposed glycoprotein analyses will provide basic information on carbohydrates known to exist on some presynaptic neurotoxins, including their mode and location of attachment, number of side-chains, and sugar identification.

With PLA_2 activity associated with all presynaptic neurotoxins, they have the potential for inducing platelet-activating factor (PAF) synthesis in endothelial cells. PAF causes blood platelet aggregation and hypotension at low concentrations (10^{-11} M) and is therefore an endogenous compound capable of causing severe physiological effects at low doses. Its induction by neurotoxins will be examined in cultured endothelial cells.

Snake venoms represent a complex mixture of proteins, many of which are involved in the breakdown and hydrolysis of biological tissues. Yet, venom is seemingly stored for indefinite periods in venom glands without either causing destruction of the venom glands or venom components themselves. This suggests that various types of natural enzyme inhibitors must co-exist in the venom to inactivate these degredative enzymes. We are hopeful that our proposed 5'-nucleotidase studies involving azido nucleotide analogs may provide us with some initial answers to this curious question. Clearly, identification and characterization of various enzyme inhibitors would be of great interest, since they might serve as potential therapeutic agents.

The molecular biology and immunology sections of the original proposal are extensions of our on-going efforts to use molecular approaches in the development of synthetic vaccines against neurotoxins. Using mutagenesis and partial gene deletion, we are hopeful that we will be able to express a non-toxic, antigenic isoform of the basic subunit of Mojave toxin that can be

used to immunize animals against rattlesnake presynaptic neurotoxins. Identification of the region of the basic subunit that promotes toxicity, may enable us to synthesize a mimicking structural peptide that is capable of generating neutralizing antibodies in animals against crotoxin and its related toxins.

Background

Earlier work in my laboratory has had a strong emphasis on rattlesnake presynaptic neurotoxins. We are interested in these toxins from several viewpoints, including their structure, mode of action, immunology and molecular biology. This proposal was an extension of earlier work, particular the molecular biology and immunology sections, but also includes studies with a number of other presynaptic neurotoxins.

Structure. We have completed the sequencing of the basic subunit of Mojave toxin (Aird, Kruggel, and Kaiser; 1990a) and the B-chain of crotoxin (Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser; 1990b). Except for the determination of the disulfide bond arrangements this completes the amino acid sequence studies on these two proteins. Collaborative work is in progress on the determination of the disulfide bond arrangements within each subunit of rattlesnake presynaptic neurotoxins using x-ray crystallography (Scott) and NMR structural analysis (Bieber).

Primary sequences have also been determined for two myotoxins from the venom of *Bothrops asper*. One, a potent PLA_2 with 67% sequence identity with ammodytoxin a, represents the first myotoxin phospholipase sequenced that lacks presynaptic neurotoxicity. Structural regions that may be responsible for neurotoxicity are proposed (Kaiser, Gutierrez, Plummer, Aird, and Odell; 1990). The second myotoxin sequenced was a lysine-49 PLA_2, which had key amino acid differences from active phospholipases (Francis, Gutierrez, Lomonte, and Kaiser; 1991a). This work demonstrates that phospholipase activity *per se* is not required in phospholipase molecules for either myotoxicity or edema inducing activities.

During our earlier purification of several snake venom presynaptic neurotoxins, we observed two proteins which were less basic than either notexin or notechis II-5, but were as toxic as notexin in the venom of the Australian Tiger snake (*Notechis scutatus scutatus*). These new toxins,

called scutoxin A and B represent isoforms of notexin (Francis, John, Seebart, and Kaiser; 1991b). Sequencing results in collaboration with Dr. Jim Schmidt (USAMRIID), indicates that scutoxin A and B differs from notexin at only two position (Francis et al., 1995c).

Crotoxin subunits cross-linked using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, were non-toxic, had reduced immunological cross-reactivity toward mono- and polyclonal antibodies raised to the basic subunit, and had lost >95% of their phospholipase activity (Lennon, Plummer, and Kaiser; 1990). Lack of a cleavable cross-linker did not permit a determination of whether the loss of toxicity was due to either subunit cross-linking *per se* or modification of essential residues.

X-ray crystallography of Mojave toxin in Keith Ward's laboratory has not yielded well defined subunit interfaces. Diffractions from the basic subunit side of intact Mojave toxin crystals have been better than those from the acidic subunit side. Poor resolution of the acidic subunit suggests that this portion of the crotoxin heterodimer assumes multiple configurations. Synthesis of useful heavy atom derivatives to improve the phase information have been unsuccessful to date. Additional crystallization and heavy-atom replacements are still ongoing in collaboration with Dr. David L. Scott in Professor Paul Sigler's group at Yale. This group had preliminary results with crotoxin crystals that were promising, but they have apparently had problems in repeating those results and obtaining satisfactory crystals. Hence their interest in Mojave toxin.

Mechanism of Action. Crotoxin binding to synaptosomes and their membranes have revealed a high affinity (K_d = 2-4 nM), specific binding site for crotoxin. Crotoxin binding was inhibited by several presynaptic neurotoxins, which were classified according to their inhibitory properties as strong (acidic subunit of crotoxin, Mojave toxin, concolor toxin, taipoxin, and pseudexin), moderate (ammodytoxin A and textilotoxin), weak (notexin and scutoxin A), very weak (notechis II-5) and non-inhibitory (basic subunit of crotoxin, β-bungarotoxin, *Crotalus atrox* and porcine pancreatic phospholipases A_2, dendrotoxin, and notechis III-4). Purified acidic subunit of crotoxin, the most potent competitor of crotoxin binding, was somewhat more competitive than intact crotoxin and the other strong inhibitors on a molar basis. Our results suggest that crotoxin and several other presynaptic

neurotoxins share common binding sites that may involve a protein on brain synaptosomal membranes (Degn, Seebart, and Kaiser, 1991). Other experiments in collaboration with Dr. Lance Simpson's laboratory (Trivedi, Kaiser, Tanaka, and Simpson, 1989), employing structurally different snake venom presynaptic neurotoxins, were carried out to determine whether neurotoxins undergo antibody escape. Preliminary results suggested that the basic subunit of crotoxin inserts into the cell membrane, but does not cross the membrane to exert its toxicity.

Immunology. Overlapping octapeptides, homologous with the complete sequence of the basic subunit of crotoxin were synthesized on polyethylene pins according to the procedure of Geysen *et al.* (1987). These peptides were used to screen our monoclonal antibodies prepared against the basic subunit of crotoxin (Kaiser and Middlebrook, 1988a,b) in an attempt to determine their antigenic determinants. Multiple assays demonstrated that none of the monoclonals recognized our solid-phase, linear peptides. When the same octapeptides were used to screen rabbit polyclonal antibodies raised to crotoxin, four regions of the basic subunit protein sequence repeatedly showed antigenicity. These were sequences 1-9, 31-44, 102-115, and 113-122, suggesting that these four regions within the basic subunit are antigenic. Four peptides corresponding to these antigenic regions were synthesized, conjugated to rabbit serum albumin, and rabbits immunized. Resulting antisera failed to neutralize against crotoxin's lethality in mice, although there was slight extension of survival times (Kaiser, unpublished data). Variations of this experiment are still being examined, because of the reported success of others in using the site-directed polyclonal antibody approach to localize the toxic site in the ammodytoxins from the venom of *Vipera ammodytes* (Curin-Serbec *et al.*, 1991).

Molecular Biology. Descriptions of the organization of genomic presynaptic neurotoxin genes is presently in its infancy, as is the expression of presynaptic neurotoxic PLA_2s *in vitro*. We have been studying the genomic structure of the Mojave toxin genes, and have now completed that work, which will be described. Expression of the subunits of Mojave toxin and generation of their native forms in *E. coli* has proven to be a more difficult experimental problem. SDS-PAGE and western blots of acrylamide gels have indicated that we are clearly expressing both subunits in all of our

expression plasmid constructs; the difficulty is in the solubilization and re-oxidation of the subunits to their native form. Our results to date will be discussed.

PURPOSE OF THE PROPOSED WORK

We used multiple approaches employing the tools of biochemistry, pharmacology, microscopy, immunology, and molecular biology to gain greater insight into presynaptic neurotoxin structure and function. Our goals were to learn more about the primary, secondary, and tertiary structure of presynaptic neurotoxins, by amino acid sequencing; screening a variety of purified toxins for the presence of natural covalent modifications such as acylation, transglutamination, and glycosylation; employ covalent modification such as enzymatic and/or chemical cross-linking of dimeric toxins to study their function; investigate an apparent new type of PLA_2 present in some elapid venoms that promotes hemorrhage and hypotension; conduct comparative studies where appropriate on other dimeric, presynaptic neurotoxins in an attempt to identify PLA_2 residues and molecular domains that promote selected biological functions; and in collaborative studies probe higher ordered structures through the use of high-resolution N.M.R. and x-ray crystallography. We wanted to determine whether the PLA_2 activity associated with all presynaptic neurotoxins had the potential for inducing platelet-activating factor synthesis in endothelial cells, and screen endogenous crude venoms and sera for various enzyme inhibitors that might serve as potential therapeutic agents; employ a photoprobe of ATP to examine crude venoms for a high affinity, specific ATP binding protein; investigate anomalous gel-filtration behavior of elapid PLA_2s and attempt to identify the reasons for this aberrant mobility. We proposed to use as tools the techniques of molecular biology and immunology to develop molecular approaches for the development of non-toxic synthetic vaccines against neurotoxins and determine critical residues in PLA_2s that promote neurotoxicity, myotoxicity, and other biological activities. We also wanted to examine the structure of genomic clones of rattlesnake presynaptic neurotoxin subunits and compare these structure with those of other organisms.

METHODS OF APPROACH

Biochemistry. a. Determine whether presynaptic neurotoxins are acylated, the extent and site(s) of acylation, and what the biochemical consequences are. b. Determine whether transglutaminases can form intra- or inter-molecular ε-(γ-glutamyl)-lysine isopeptide bonds in crotoxin and the resulting affects on phospholipase activity, neurotoxicity, and immunoreactivity. c. Examine more closely and characterize the acidic proteins originally observed in *Notechis scutatus scutatus* venom. d. Screen a variety of venoms for glycoproteins and analyze the carbohydrate moieties present. e. Prepare an enzymatically cleavable protein cross-linker. Use the linker or a chemically cleavable cross-linker to determine whether or not crotoxin subunit dissociation is essential for neurotoxicity. f. Provide Dr. Allan Bieber (Arizona State University) with sufficient Mojave toxin acidic subunit for 2-D and 3-D NMR investigations. g. Determine whether platelet-activating factor concentration is increased in endothelial cell cultures when exposed to presynaptic neurotoxins. h. Further examine the mechanism of action of the PLA_2 neurotoxins using the tools available in various disciplines, including biochemistry, chemistry, pharmacology, and microscopy. i. Compare and contrast the properties of other heterodimeric neurotoxins that may come available with those of the rattlesnake presynaptic neurotoxins in an effort to gain greater insight into the functional roles of different residues and molecular regions. For example, how do these heterodimeric toxins associate?

Molecular Biology. a. Develop a system in *E. coli* for the expression of Mojave toxin subunit cDNAs. Use this system to generate non-toxic, immunogenic forms of the basic subunit for immunization studies. Also use the expression system to generate site-specific mutant proteins of Mojave toxin for structure-function studies. b. Investigate the organization of the genomic clones of rattlesnake presynaptic neurotoxins and compare their structures with others available.

Immunology. a. Attempt to identify the toxic and other functional sites of rattlesnake presynaptic neurotoxins. b. Initiate studies using murine antibody fragment clones specific for Mojave toxin in a bacteriophage λ immunoexpression library to determine their utility with neurotoxins.

Exploratory studies. a. Determine whether inhibitors for degredative enzymes exist in snake venom and carry out as much characterization as possible. b. Examine the reasons for the anomalous gel-filtration observed during the fractionation of N. s. scutatus venom (Francis et al., 1991b). c. Use an azido-ATP photoprobe to screen a venom(s) for the presence of a protein with a specific, high-affinity ATP-binding site.

BODY OF REPORT
Methods, Results, and Discussion

a. Biochemistry.

1. During our PURIFICATION OF NEUROTOXIC PROTEINS from crude *Notechis scutatus scutatus* venom, we encountered a set of acidic proteins which were toxic when injected i.v. in mice at ≈1 μg/g. These proteins promoted severe hemorrhaging when injected either i.v. or i.p. in mice, induced a rapid loss in blood pressure in mice when injected i.v., and displayed phospholipase activity against the small PLA_2 substrate NOB (4-nitro-3-octanoyloxybenzoic acid) as well as phosphatidylcholine. [Initial studies indicated no phospholipase activity was associated with these acidic proteins, which was incorrect.] These proteins also exhibited apparent molecular weights in the 18,000-21,000 range based on mobility in SDS-PAGE, slightly higher than the neurotoxin phospholipases (13,000-15,000). In addition, polyclonal antibodies raised to one of these acidic proteins recognized not only the other acidic proteins, but also notexin, scutoxin and notechis II-5, suggesting that the acidic proteins had antigenic regions similar to those in the neurotoxic phospholipases. Thus, it appeared that the acidic proteins could be PLA_2-like proteins which were modified in some way to appear to have higher apparent molecular weights. We therefore examined these proteins in some detail for unusual structural modifications.

One publication (Francis, Williams, Seebart, and Kaiser, 1993b) was published describing this group of toxic acidic proteins called $HT_{a\ to\ i}$. A polyclonal antibody raised against one of the proteins in the group HT_g, binds to other purified proteins suggesting that they are isoforms of the same protein. Many other elapid crude venoms contain proteins which recognize

the polyclonal antibody raised against HT_g. Crotalid and viperid crude venoms do not recognize this antibody although some of their component proteins are known to exhibit hypotensive and hemorrhagic activities. A combination of gel-filtration on Sephacryl S-200, cation-exchange and anion-exchange chromatography allows isolation of the *N. s. scutatus* proteins in high purity. They are the first hypotension inducing proteins to be purified from an Australian elapid.

2. A NEW TYPE OF TOXIC SNAKE VENOM PHOSPHOLIPASE A_2 WHICH PROMOTES HYPOTENSION AND HEMORRHAGE IN MICE. Phospholipase A_2s (PLA_2s) isolated from snake venoms have generally been described as neurotoxic (acting presynaptically at neuromuscular junctions), myotoxic, cardiotoxic or non toxic as demonstrated by toxicity assays conducted by i.v., i.m. or i.p. injections in mice (Rosenberg, 1990). PLA_2s which are non toxic may exhibit a biological activity, such as platelet aggregation or hemolysis, but this activity may not be lethal to mice. We have recently identified a new type of toxic PLA_2 that is present in some elapid snake venoms. After injection of these toxins i. v. in mice, hemorrhage is observed in several tissues, but is most evident in the lungs where blood is observed in the thoracic cavity. After i.p. injection hemorrhage is observed in the peritoneal cavity. In cases studied to date, a rapid loss of blood pressure is also observed. Based on these pharmacological activities, we are calling these proteins hypotensive/hemorrhagic (HT) PLA_2s.

i) HEMORRHAGIC/HYPOTENSIVE PLA_2S IN TIGER SNAKE VENOM. Hemorrhagic/hypotensive PLA_2s were first encountered during purification of neurotoxins from the venom of the Australian tiger snake (*Notechis scutatus scutatus*). When the crude venom fraction obtained from a gel filtration column which contains neurotoxic PLA_2s [S200 fraction IIB, see Fig. 1 in Francis et al., 1991b] is applied to a Mono S cation exchange column, basic neurotoxins bind to the column but a number of acidic proteins migrating on SDS-PAGE with 18,000-21,000 apparent mol. wts. are collected in the flow through fraction. As a whole these acidic proteins are moderately toxic when injected i. v. in mice. Individual proteins in this group, called HT_a to HT_i, are separated by Mono Q chromatography and correspond to peaks 2 to 10 in Fig. 1 (see APPENDIX). They show i.v. LD_{50}-values in mice in the 1-2 μg/g range, approximately 200-fold higher (less toxic) than neurotoxins such as notexin (Francis et al., 1993b). A polyclonal

antibody raised against HT_g recognizes the other toxic acidic proteins and also the neurotoxins, notexin, scutoxin [an isoform of notexin (Francis et al., 1995a)], and notechis II-5. HT_a-HT_i are probably isoforms of the same protein. This antibody binds to proteins in a number of other Australian elapid venoms and also proteins in the venoms of the coral snake, *Micrurus frontalis frontalis*, the cobras *Naja naja atra* and *Naja nigricollis*, and the krait, *Bungarus multicinctus*, but not to proteins in the venom of the mamba, *Dendroaspis angusticeps*, or to proteins in viper and rattlesnake venoms.

The behavior of HT_a-HT_i on SDS-PAGE is unusual. In solubilizing solution lacking DTT, these proteins migrate with apparent mol. wts. ranging from 16,000 to 21,000. In the presence of DTT, their mobilities differ, but are in the same molecular weight range. Proteins in some Mono Q peaks contain more than one protein, as expected from the Mono Q elution profile which suggests that complete separation of the isoforms had not been accomplished. Phospholipase A_2 activity is observed with these proteins using either micellar phosphatidylcholine or nonmicellar 4-nitro-3-octanoyloxybenzoic acid as substrate. Therefore, these proteins are PLA_2s which have unusually high mol. wts. as measured by SDS-PAGE (Francis et al., 1995b).

Amino acid sequencing of HT_e has demonstrated that it is a group I PLA_2 (Fig. 2) but that it contains in its sequence amino acids 62-66 [using the numbering system of Renetseder et al., 1985] which are present in pancreatic PLA_2s, but are normally missing in elapid snake venom PLA_2s. The only PLA_2s from snake venoms that have been found to contain these amino acids are the non-toxic OS1 PLA_2 from *Oxyuranus scutellatus* venom (Lambeau et al., 1995), and the γ-subunit of taipoxin and the D-subunit of textilotoxin, both of which are weakly PLA_2 active and non toxic (Fohlman et al., 1977; Pearson et al., 1991). These sequences are shown in Fig. 2. Unlike HT_e and HT_g, the taipoxin and textilotoxin subunits have additional amino acids at their N-terminal ends like pancreatic proPLA_2s, additional cysteines in their amino acid sequences, and are glycosylated. A partial sequence of HT_g shows that it contains a sequence in the segment containing the "pancreatic loop" residues almost identical to that of HT_e (Fig. 2). The presence of the pancreatic loop may explain the large apparent mol. wts. of these proteins on SDS-PAGE since porcine pancreatic PLA_2 itself migrates with an apparent mol. wt. of $\approx$20,000 in the absence of reducing agent.

The N-terminal 57 amino acids and C-terminal 25 amino acids of HT_e show extensive homology with neurotoxins isolated from *N. s. scutatus* venom, which explains their cross-reactivity with the HT_g polyclonal antibody. For example, HT_e has 48 and 20 amino acids identical with notechis II-5 in the N-terminal and C-terminal segments, respectively. The central segment between amino acids 58 and 100 shows low homology with other *N. s. scutatus* PLA_2s whether they are non-toxic, such as notechis II-1 (Lind and Eaker, 1980) and notechis 11'2 (Bouchier et al., 1991) or neurotoxic like notexin (Halpert and Eaker, 1975; 1976). In this segment greatest homology is found with OS1 (34 identical residues out of 43) and taipoxin-γ (31 identical residues). Overall, HT_e has highest homology with a PLA_2 from *Bungarus fasciatus* venom (86 identical residues; Liu et al., 1989), but this PLA_2 does not have the pancreatic loop residues (Fig. 2) and is not reported to be toxic.

A curious observation which we have not fully investigated is that extensive dialysis of several of these toxins against water leads to a change in their elution behavior from the Mono Q column. They elute at a lower concentration of sodium chloride ($\approx$0.025M) than they do on the first chromatographic separation. A possible explanation for this apparent decrease in acidity is that these proteins are non-covalently associated with small, negatively charged molecules which are removed during the dialysis. We have described the anomalous behavior of notexin and scutoxin on gel filtration columns in the presence of different anions and this behavior is best explained by non-covalent association of these proteins with anions (Francis et al., 1995c). Another possible explanation might be that they are covalently modified at lysine or arginine and these modifications are hydrolyzed during dialysis producing more basic proteins. However, evidence for lysine/arginine modification is not observed either by mass spectrometry or during amino acid sequencing of HT_e. We have not yet examined whether the toxicities and PLA_2 activities of these proteins are altered following extensive dialysis.

Injections of the acidic proteins at 1-2 μg/g either i.v. or s.c. cause severe pulmonary hemorrhage and edema, possibly due to pulmonary vascular damage. In addition, mice injected i.v. show acute endomyocardial and focal hepatic necrosis, probably via ischemia. At a dose of 4 μg/g i.v., acute tubular nephrosis is also observed. Mice injected s.c. show little hemorrhage at the injection site. Severe hemorrhaging of the lungs is observed in mice

injected i.p. with 5 μg/g of either HT_e or HT_g. Red colored urine is frequently observed, indicating hemolysis. Hemorrhage resulting from these PLA_2s is clearly different from that produced by most other venom hemorrhagins. Hemorrhagic toxins in rattlesnake and vipers vary in mol. wt. from 15,000 to 100,000. Some have been well characterized and almost all possess proteolytic activity (Bjarnason and Fox, 1988-89; Tu, 1991). They show hemorrhage around the site of s.c. injection (Spencer and Kaiser, 1996). A group II PLA_2 which appears to induce lung hemorrhage and kidney and liver damage in a similar manner to the *N. s. scutatus* toxins is *Vipera russellii* PL-VIIIa (Kasturi and Gowda, 1989).

Extraction of blood from these mice shortly after toxin injection is difficult, suggesting that their blood pressure has been markedly reduced. When the effects of these toxins on blood pressure are measured following administration of 1.5 μg/g i.v., a rapid decrease is observed in the first few min (Francis et al., 1993b). Blood pressure returns to nearly the original level after 10 min. These mice died within 2-2.5 hr. Since whole venom from a coral snake, Micrurus fulvius fulvius, had previously been shown to cause a decrease in blood pressure in dogs (Weis and McIsaac, 1971; Ramsey et al., 1972), we injected venom from another coral snake, *M. f. frontalis*, to see if we could observe a similar decrease in blood pressure. At a concentration of 0.5 μg/g, a decrease is indeed observed, but the decrease is not as rapid as that observed for the *N. s. scutatus* toxins and the recovery is slower (Francis et al., 1993b). Injections at this concentration are not lethal to mice. The rapid effect suggests that the decrease in blood pressure is probably not just a result of hemolysis. During the first few minutes of the blood pressure determinations with the *N. s. scutatus* toxins, there is also a change in heart beat, as shown by the pulse transducer, from a regular to a highly erratic pattern. This suggests that the toxins may have a direct effect on the heart, but this has not yet been confirmed. Crude *M. f. frontalis* venom did not affect the heart beat as strongly as the purified *N. s. scutatus* proteins.

Although proteins such as HT_e and HT_g are toxic, their LD_{50}-values are far higher than those of neurotoxins such as notexin and their pathologies indicate that they may not be neurotoxic. Therefore, neuropharmacological and neurophysiological studies have been performed to determine whether HT_e is neurotoxic. HT_e produces concentration-dependent blockade of

neuromuscular transmission, characteristic of PLA_2s acting preferentially on nerve endings by blocking stimulus-evoked transmitter release. However, neuromuscular blockade by HT_e is accompanied by loss of nerve and muscle membrane potentials and by a loss of ability to respond to direct stimulation. The fact that muscle cannot be depolarized by direct stimulation and cannot propagate impulses argues that HT_e does not act selectively on nerve endings. Rather, HT_e exerts pathologic effects both presynaptically and postsynaptically. At the same time that the toxin is producing loss of neuromuscular transmission, it is also causing profound blanching of muscle. This effect, which in part must be due to loss of myoglobin, suggests that the toxin acts on muscle as well. Thus, HT_e appears to act on both nerve and muscle (Francis et al., 1995b).

Why does HT_e behave differently from notexin? Why does it not show preferential action on nerve endings? HT_e is homologous with notexin in the C-terminal region where chemical modification of a tryptophan causes loss of toxicity (Mollier et al., 1989), and where amino acids are partly responsible for ammodytoxin toxicity [see articles by Krizaj et al. (1989) and Curin-Serbec et al. (1991)]. In addition, the N-terminal sequence shows high homology with the neurotoxins. The principal difference is in the ß-wing and the pancreatic loop. On the basis of amino acid sequence homologies, Dufton and Hider (1983) suggested that the ß-wing is important in neurotoxicity of elapid PLA_2s. It appears that its action as a neurotoxin is impaired by the ß-wing/pancreatic loop changes, perhaps by altering their interfacial binding to membranes and/or perhaps by changing receptor recognition.

ii. HEMORRHAGIC PLA_2S IN CORAL SNAKE VENOMS. When considering which other venoms might contain similar types of PLA_2s, we thought that *M. f. frontalis* venom would be a promising candidate since it reduces blood pressure in mice and contains proteins of 21,000 apparent mol. wt. recognized by the HT_g antibody. Fractionation of *M. f. frontalis* venom by gel filtration produces 10 peaks of absorbance (Fig. 3). Peak 10 largely contains guanosine. Peaks 5, 6, and 7 are toxic to mice with i.v. LD_{50}-values of <1 $\mu g/g$, ≤ 2 $\mu g/g$, and ≈ 0.7 $\mu g/g$, respectively; and phospholipase activities of 120, 500, and 944 μmole fatty acid released $min^{-1}mg^{-1}$ protein, respectively. Each of these peaks contains proteins in the 18,000 to 22,000 mol. wt. range as shown by SDS-PAGE (Francis et al., 1996b). We have further fractionated peak 5 using ion exchange chromatography. With Mono Q,

four major peaks of bound material are collected (Fig. 4), and with Mono S, 11 peaks of bound material were observed (Fig. 5). Mono S peak 1 does not appear to contain protein. Mono Q peak 4 and Mono S peaks 9 and 11 are PLA_2-active with apparent mol. wts. of ≈21,000. PLA_2s in Mono Q peak 4 and Mono S peak 9 produce hemorrhage in the lungs when injected i.v. in mice with doses of 0.5-1.0 μg/g, closely resembling the effects observed with HT_a-HT_e (Francis et al., 1996a and b). However, Mono S peak 11 (Fig. 5) contains a very basic, although similar PLA_2-active protein which is non toxic at ≈4 μg/g. The diet of this coral snake includes other snakes (mainly colubrids) and amphisbaenians (Jorge da Silva, 1995). It is possible that the PLA_2s which are non-toxic to mice are toxic to other animals (Daltry et al., 1996).

Proteins which do not bind to the Mono Q column are recognized by anti-HT_g polyclonal antibodies. Of the proteins that bind, only those in peaks 1 and 2 in Fig. 4 are recognized and at that very weakly. From the Mono S profile shown in Fig. 5, only proteins in peaks 9 and 11, and a minor component of peak 10 are recognized by the anti HT_g antibodies. No cross-reactivity of *M. f. frontalis* proteins is observed with a monoclonal antibody raised against pseudexin or a polyclonal antibodies against ß-bungarotoxin. However, a protein of ≈21,000 apparent mol. wt. in S-200 peak 7 (Fig. 3) is recognized by a polyclonal antibody against notexin. Surprisingly, we have not yet found in *M. f. frontalis* venom, PLA_2s of the "normal" elapid type which typically migrate on SDS-PAGE in the 13,000-16,000 range.

Unusual chromatographic behavior has also been observed during purification of *M. f. frontalis* PLA_2s. When peak 5 recovered from the S-200 gel filtration column (Fig. 3) is dialyzed against water, lyophilized, and applied to a Mono Q column, a number of proteins do not bind, and bound proteins elute in four major peaks (Fig. 4). When proteins which do not bind to the Mono S column are dialyzed against water, lyophilized, and applied to the Mono Q column under identical conditions to those used to fractionate the whole of peak 5, a chromatogram is obtained which is different. We have not yet found an explanation for this behavior. One possibility is that the change is produced by dissociation of a small molecule during Mono S chromatography.

More detailed histological analyses show that Mono S peak 9 (Fig. 5) and Mono Q peak 4 (Fig. 4), when injected i.v. produced perivascular

hemorrhage in the lungs with hemorrhagic lesions, increasing in number with time. These findings suggest that the erythrocytes first escape via the junction of the endothelial lining and later as a result of the disruption of the basement membrane. Microscopic hematuria was evident after 5-10 minutes following the injection, evolving to hemoglobinuria, as an acute toxic nephritis (Jorge da Silva et al., 1996).

A number of other coral snake crude venoms injected into mice produce hemorrhaging of lungs and kidney damage similar to *M. f. frontalis*, including *Micrurus spixi, Micrurus frontalis altirostris, Micrurus frontalis brasiliensis, Micrurus baliocoryphus, Micrurus pyrrhocryptus*, and *Micrurus corallinus* that feed on other snakes and amphisbaenians. Venom from *Micrurus lemniscatus*, a snake that feeds on other snakes and fish produces less lung and kidney damage than *M. f. frontalis* venom. A weak effect is observed with venoms from *Micrurus decoratus*, *Micrurus albicinctus* (diet unknown) and *Micrurus hemprichii* that feeds primarily on *Peripatus* (Onychophora), and effects are completely absent with *Micrurus surinamensis* that feeds on fish (Jorge da Silva, 1995; Jorge da Silva et al., 1996). Consequently, one of the principal toxic mechanisms of *M. f. frontalis* venom and perhaps other coral snake venom toxic PLA_2s may be through this hypotensive/hemorrhagic mechanism in higher vertebrates.

We suspect that the hypotensive/hemorrhagic type of PLA_2 may be widely distributed in elapid venoms. A number of other elapid venoms/venom proteins promote hypotension in addition to coral snake venoms. Most studied are those from cobras (Lee and Lee, 1979). Certain cobra venom PLA_2s have been reported to promote short-lived decreases in blood pressure (Fletcher et al., 1981). Examples of other Australian elapid venoms which produce a sudden fall in blood pressure are the black snake, Pseudechis porphyriacus (Martin, 1895; Trethewie and Day, 1948) and the Australian copperhead, *Denisonia superba* (Kellaway and Le Messurier, 1936; Feldberg and Kellaway, 1937). The most studied group II phospholipases A_2 that promote hypotension are those from *Vipera russellii* venom (Huang, 1984). As mentioned previously, one of these, PL-VIIIa, induces neurotoxic symptoms and damages organs such as lung, liver and kidney (Kasturi and Gowda, 1989). Elapid venoms are generally thought to be non-hemorrhagic. However, *Ophiophagus hannah* (king cobra) venom contains toxins which promote hemorrhage in rabbits and hares but not in mice, rats or guinea-pigs (Weissenberg et al., 1987). As mentioned before, our studies show that

several elapid venoms contain proteins which bind to the HT_g antibody (Francis et al., 1996a and b).

The particular structural features of HT PLA_2s that are required for their biological effects need to be determined. N-terminal amino acid sequences of a few coral snake PLA_2s have been reported but none have proceeded past residue 38 (Possani et al., 1979; Mochca-Morales et al., 1990; Takasaki et al., 1991). It will be of considerable interest to obtain the full sequence of HT PLA_2s from coral snakes (sequencing of one of these is currently in progress; Francis, Schmidt, and Kaiser, unpublished data), and perhaps similar PLA_2s in other elapid venoms, to discover amino acids which are in common with those found in HT_e and HT_g. If so, it will carry the strong implication that these residues are important in exerting pathological activity. Sequencing of non- toxic HT PLA_2-like proteins, such as that in Mono S peak 11 (Fig. 5A) from *M. f. frontalis* venom, will also provide valuable comparative information, since sequence differences may be responsible for the differences in toxicity levels observed in mice. Sequence differences may also be responsible for toxicity differences observed in different species, such as with king cobra venom (Weissenberg et al., 1987).

iii) POSSIBLE INVOLVEMENT OF HT PLA_2S IN PROMOTING SEPTIC SHOCK. An unanswered but extremely interesting question is how do these HT PLA_2s exert their toxic effects? Their pathology closely resembles that observed in septic shock syndrome which is characterized by refractory hypotension and progressive organ damage leading to death, a condition that is often accompanied by hemorrhage (Rothstein and Steiner, 1988; Dinarello, 1991; Parrillo, 1993; Natanson et al., 1994). Recently, a possible relationship between pancreatic PLA_2s and septic shock has been demonstrated. A high affinity receptor for pancreatic PLA_2 and some venom PLA_2s has been identified in a number of cells (Hanasaki and Arita, 1992; Ishizaki et al., 1994; Lambeau et al., 1994; Kishino et al., 1994; Ancian et al., 1995; Lambeau et al., 1995). This receptor is structurally related to the Ca^{2+}-dependent (C-type) animal lectin family of receptors typified by the mannose receptor of macrophages (Taylor et al., 1990). Binding of pancreatic PLA_2 to the receptor occurs via sugar residues on the receptor and in the presence or absence of Ca^{2+}, showing that it is independent of PLA_2 activity (Ishizaki et al., 1993; Lambeau et al., 1995). Comparisons of the binding of a number of

mutant pancreatic PLA_2s have shown that the region involved in binding, includes Ca^{2+}-binding loop residues but not the pancreatic loop (Lambeau et al., 1995). In the case of mesangial cells, binding of pancreatic PLA_2 to its receptor induces release of prostaglandin E2 and an endogenous group II PLA_2. Tumor necrosis factor alpha and interleukin 1ß are cytokines that induce similar responses by these cells to that induced by pancreatic PLA_2. Since these cytokines play critical roles in the promulgation of septic shock syndrome in response to infectious agents (reviewed in Goldblum, 1991), it has been suggested that pancreatic PLA_2 might have a function in the development of inflammatory conditions (Kishino et al., 1994). Given the structural similarities of HT PLA_2s with pancreatic PLA_2, it is possible that the HT PLA_2s also bind to a receptor (not necessarily the same receptor), inducing the release of pro-inflammatory mediators. This binding may be responsible for the rapid effects of HT PLA_2s on blood pressure and heart beat. Whether or not HT PLA_2 toxicity requires PLA_2 activity as well as receptor binding needs to be established. Clearly, HT PLA_2s are promising models to understand certain aspects of septic shock and since the deaths from septicemia are high [175,000 per year in the U. S. A. (Stone, 1994)], HT PLA_2 studies may yield valuable insights into new possibilities for treatment of these conditions. Group II PLA_2s such as *V. russelli* PL-VIIIa that produce organ damage (Kasturi and Gowda, 1989) may be acting in a way similar to the endogenous group II PLA_2 that is elevated in concentration in the serum of patients with septic shock (Vadas et al., 1992 and 1993). Consequently, particular group II PLA_2s may also prove useful in the study of proinflammatory conditions.

The above section 2 of this Final Report served the basis for an invited review chapter submitted to Dr. G. S. Bailey for inclusion in his upcoming book *The Enzymology of Snake Venoms*, which will be published in late 1996 or early 1997. It is cited in the LITERATURE CITED section as Francis, Jorge Da Silva, and Kaiser (1996a).

iv) POSTSYNAPTIC NEUROTOXINS IN CORAL SNAKES. One set of proteins purified from the venom of the Brazilian coral snake, *Micrurus frontalis frontalis* had molecular weights as shown by SDS-PAGE in the 8-13 kDa range. This set of proteins included some proteins which are toxic to mice and others which are not. One of these basic proteins was purified by a combination of gel filtration on Sephacryl S200 and cation-exchange chromatography on Mono S. It possessed an i.v. LD_{50}-value of 0.25-0.5µg/g in

mice, which exhibited neurotoxic symptoms following injection. This peak was reduced, alkylated, and the first 35-residues of the N-terminal sequence determined to give the primary sequence shown in Fig. 5B. Selected residues found in this partial sequence show complete sequence identity with known conserved residues found in short postsynaptic neurotoxins (Endo and Tamiya, 1991). For additional details on the postsynaptic toxins from *M. f. frontalis*, see the paper by Francis et al. (1996b).

3. N.M.R. ANALYSIS. Our collaborative effort with Dr. Allan Bieber (Arizona State University) on the high-resolution NMR structural analysis of the acidic subunit, have made us better appreciate the complexity of the native acidic subunit. (This probably explains why the x-ray crystallographers have not been able to get high-resolutions crystals from Mojave toxin provided to them.) There are clearly numerous isoforms of the acidic subunit, most of which are probably generated by different degrees of processing of the three peptides by proteases in the conversion of pro-acidic subunit to the mature form. I have enclosed a 'typical' mass spectrum (Fig. 6) of the acidic subunit of Mojave toxin obtained by Bieber that shows the multiple isoforms. Attempts by Bieber's laboratory to further fractionate the acidic subunit isoforms into a single component using reversed-phase chromatography was unsuccessful. We extended their separation experiments using a combination of ion-exchange and reversed-phase chromatography in an attempt to isolate a 'single, major isoform'. Ideally, we need a single isoform to carry out the NMR work to allow data interpretation. It's interesting that the basic subunit of Mojave toxin appears much more homogeneous than the acidic subunit as seen in its 'typical' mass spectrum (Fig. 7). There could of course be amino acid substitutions in different isoforms, but if their molecular weights are similar they would not necessarily be resolved by the analysis.

We prepared 10-15 mg of 'highly purified' acidic subunit of crotoxin for Bieber, using a brute force approach which involved repetitive chromatography runs on multiple columns. The sample we provided is 'highly enriched' in a major isoform of the acidic subunit, but not completely homogeneous as determined by mass spectroscopy (see Fig. 8). There is one predominant peak (m/z =9472), with what appear to be sinapinic acid adducts (mol. wt.=208), at m/z of 9681 and 9889. There are traces of the basic subunit at 14280 and intact toxin at 23685, as well as acidic subunit dimer

(18987) and trimer (28527). We are hopeful that the sample will provide us at least some preliminary insights into the structure of the acidic subunit of the rattlesnake presynaptic neurotoxins. These analyses are currently in progress.

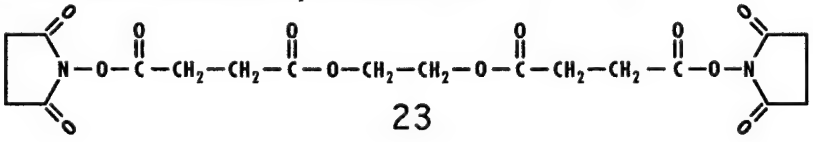
4. X-RAY STUDIES. After discussions with Dr. Keith Ward at the Naval Research Laboratory and Dr. David Scott, in Professor Paul Sigler's x-ray diffraction group at Yale, we purified about 25 mg of Mojave toxin and sent it to Scott. This "last gasp effort" was an attempt to salvage a lot of work and effort originally carried out in collaboration with Dr. Keith Ward on the x-ray crystallography solution of crotoxin/Mojave toxin structure. Unfortunately, Scott has been unable to grow crystals large enough to be useful in heavy-atom replacement studies which are required to solve the structure. Until new technologies are available, this problem should probably remain on the shelf.

5A. After preliminary investigations, we discontinued our work on the enzyme 'DESTABILASE' from leech saliva. Our source of saliva from Accurate Chemical and Scientific Corp. did not prove satisfactory. We found low protein content in the saliva from Accurate (≈60μg of protein/ml) and no esterase activity on a synthetic chromogenic substrate that had been shown by others to be hydrolyzed by 'destabilase'

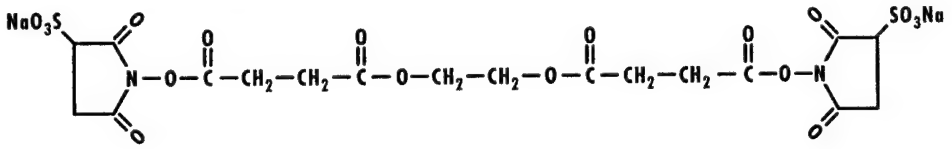
We were unable to obtain saliva from medicinal leeches (*Hirudo medicanalis*) in our laboratory. Because a satisfactory supply of leech saliva was unavailable, we directed our attention to chemically cleavable cross-linkers. Work with them is described below. Future work with the enzyme 'DESTABILASE' is potentially very interesting, if a satisfactory supply of the enzyme or saliva becomes available in the future.

5B. CHEMICALLY CLEAVABLE PROTEIN CROSS-LINKERS. Once we discontinued our work with the enzymatically cleavable cross-linkers, we directed our attention to the chemically cleavable molecules. After focusing on several of these, we concentrated our attention on two. Namely, EGS and sulfo-EGS (ethylene glycol *bis*[succinimidylsuccinate]) and its sulfonated analog, whose structures are shown below.

EGS (m.w. 456.4 with a spacer arm of 16.1 Å)



Sulfo-EGS (m.w. 660.5)



Both of these molecules are homobifunctional NHS-ester cross-linkers, which can be cleaved by incubation with high concentrations of hydroxylamine. Our overall strategy was to react the isolated acidic subunit of crotoxin with a large excess of cross-linker. Following acidification, excess cross-linker and 'activated acidic subunit' was separated by gel filtration on Sephadex G25. Recovered 'activated acidic subunit' was combined with stoichiometric amounts of the basic subunit of crotoxin, the pH adjusted to ≈8, and the reaction allowed to proceed for several hours. The product contains the desired cross-linked, in addition to free acidic and basic subunits.

Previous problems encountered earlier, resulted from attempts to cross-link the acidic and basic subunits of crotoxin/Mojave toxin, in the intact toxin directly. Since EGS reacts with nucleophiles (ε-amino groups of lysine), the basic subunit has about a 3 to 1 ratio of reactive lysine groups relative to the acidic subunit. It therefore reacts more rapidly and extensively than the acidic subunit, giving an excessive number of half-reacted EGS cross-linkers. For more details, see the paragraph and data below.

We optimized conditions for cross-linking the two subunits of Mojave toxin/crotoxin with the cleavable cross-linker EGS. Acidic subunit is first reacted with EGS for 2 min at room temperature at pH 8. The reaction is stopped by adjusting the pH to 5.5-6.0 with H_3PO_4 and passed over a small Sephadex G25 column at pH 6. Recovered acidic subunit should be partially reacted with one end of the EGS cross-linker. To this fraction, the basic subunit of Mojave toxin is added and the pH adjusted to pH 7.5 and then incrementally to 8 over the next 3 hr at room temperature. Finally, the pH is adjusted to 6 and the reaction mixture separated over a small G25 column once again. The high mol. wt. sample recovered from the G25 column was dialyzed against dH_2O and lyophylized. It was dissolved in 50mM Tris-HCl (pH 7.4)-6M urea and applied to a (0.5 x 5 cm) Mono Q column and eluted with a linear gradient of 1M NaCl in the above Tris-urea buffer. Dissociated basic

subunit was unbound to the column eluted early during the initial wash with Tris-urea. Cross-linked toxin eluted in a group of about 6 poorly resolved peaks between 0.18-0.25M NaCl. Free acidic subunit eluted later in multiple peaks between 0.30-0.37M NaCl (data not shown).

Cross-linked toxin peaks were pooled, dialyzed against dH_2O and lyophylized. It was redissolved in 50mM Tris-HCl (pH 7.6)-6M GuHCl and applied to a 1 x 30 cm Superdex 75 column and eluted at a flow rate of 0.3 ml/min. A typical gel-filtration run is shown in Fig. 9. Control experiments suggested that the first eluting, symmetrical peak corresponded to cross-linked crotoxin and the second peak with a shoulder on the late-eluting side corresponded to acidic subunit. Cross-linked crotoxin was pooled, dialyzed against dH_2O, and lyophylized. An aliquot was treated with hydroxylamine, dialyzed against dH_2O, and lyophylized. Samples of cross-linked crotoxin treated in various ways, as well as other samples were run on SDS-PAGE and silver stained. Results are shown in Fig. 10. Preliminary characterization experiments of the cross-linked crotoxin indicate that both the cross-linked crotoxin and hydroxylamine-treated cross-linked crotoxin retain phospholipase activity. Toxicity determinations and other forms of characterization are in progress.

In the absence of reducing agent, a prominent, new band appears on the silver-stained 15% PAG, migrating at molecular weight of ≈25,000 (Fig. 10, lanes 3 and 4). This would be the expected mol. wt. of an intact molecule of Mojave toxin consisting of an acidic and basic subunit. Following recovery from Superdex 75 the apparent cross-linked toxin appears highly purified (lane 4). Treatment of the cross-linked material with hydroxylamine results in a reappearance of the protein migrating in the areas expected for acidic and basic subunits (lane 5). Treatment of products generated by hydroxylamine results in the loss of acidic subunit, as expected (lane 9). This new band disappears when the sample is treated with reducing agent (DTT), as expected if the cross-linking has occurred between one of the peptides making up the acidic subunit and the basic subunit. Based on these results, plus the elution position on Superdex 75 (Fig. 9), we are convinced that we have successfully cross-linked the subunits of crotoxin. With the hydroxylamine-cleavable cross-linker, we are now in a position to answer the question of whether the two subunits need to separate to be toxic.

Once I complete this report, I intend to return to the laboratory and complete the preliminary characterization of this cross-linked material. We plan to write up a short communication of these results for *Toxicon* in the next month or so.

6. MECHANISM OF ACTION OF PHOSPHOLIPASE A_2 NEUROTOXINS. Phospholipase A_2 neurotoxins act at the neuromuscular junction to block release of acetylcholine (Rosenberg, 1986, 1990). The full details of toxin-induced blockade of exocytosis are not known, but at least two steps appear to be involved. During the initial step, the toxins bind to specific sites on the cell surface, and for certain PLA_2 neurotoxins, some of the binding sites have been determined. For example, β-bungarotoxin, crotoxin and perhaps other PLA_2 toxins bind to voltage-dependent potassium channels (Dreyer, 1990; Strong, 1990). This binding does not produce neuromuscular blockade.

During a subsequent step, PLA_2 neurotoxins produce complete neuromuscular blockade. This step is presumed to be enzymatic in nature and it is related to cleavage of the *sn-2* ester linkage of glycerophospholipids (Chang, 1985). It has not been determined whether all PLA_2 neurotoxins act on the same substrate or whether each neurotoxin has its own substrate. It has also not been established how cleavage of one or more phospholipids can produce neuromuscular blockade.

One of the major obstacles to determining the full mechanism of PLA_2 neurotoxin action has been the difficulty of identifying the sites at which they localize to produce their poisoning effects. Our microscopy studies described below, have attempted to gain morphological evidence to resolve the issue of internalization. In collaboration with Dr. Lance Simpson's laboratory, we also employed pharmacological experiments that were combined with physical chemistry experiments to identify the site at which PLA_2 neurotoxins localize to produce their poisoning effects. The results suggest that the PLA_2 neurotoxins are not endocytosed to exert their effects. Instead, these toxins bind to the cell surface and then undergo a molecular rearrangement that gives them access to substrates in the membrane. It is at this site that the toxins act to produce blockade of neuromuscular transmission.

i. PHARMACOLOGICAL AND PHYSICAL CHEMISTRY STUDIES. Experiments were conducted on mouse hemidiaphragm preparations using five PLA_2 neurotoxins of differing chain structures and antigenicities [notexin(one chain); crotoxin (two chains not covalently bound), β-bungarotoxin (two chins covalently bound); taipoxin (three chains), and textilotoxin (five chains; one copy each of three chains and two copies of a fourth chain)]. Three clostridial neurotoxins (botulinum neurotoxin types A and B, and tetanus

toxin) were used in comparison experiments. PLA_2 neurotoxins produced concentration-dependent blockade of neuromuscular transmission. There was no obvious relationship between chain structure and potency, but there was an indication of a relationship between chain structure and binding. The binding of notexin was substantially reversible, the binding of crotoxin was slightly reversible, and the binding of β-bungarotoxin, taipoxin and textilotoxin was poorly reversible. Experiments with neutralizing antibodies indicated that PLA_2 neurotoxins became associated with binding sites on or near the cell surface. This binding did not produce neuromuscular blockade. When exposed to physiological temperatures and nerve stimulation, bound toxin disappeared from accessibility to neutralizing antibody. This finding suggests that there was some form of molecular rearrangement. The two most likely possibilities are: (1) there was a change in the conformation of the toxin molecule, or (2) there was a change in the relationship between the toxin and the membrane. The molecular rearrangement step did not produce neuromuscular blockade. At a later time there was onset of paralysis; the amount of time necessary for onset of blockade was a function of toxin concentration. PLA_2 neurotoxins were not antagonized by drugs that inhibit receptor-meditated endocytosis. In addition, PLA_2 neurotoxins did not display the pH-induced conformational changes that are typical of other endocytosed proteins, such as clostridial neurotoxins. However, PLA_2 neurotoxins were antagonized by strontium, and this antagonism was expressed against toxins that were free in solution and toxins that were bound to the cell surface. Limited antagonism was expressed after toxins had undergone molecular rearrangement, and no antagonism was expressed after toxin-induced neuromuscular blockade. The cumulative data suggest that PLA_2 neurotoxins are not internalized to produce their poisoning effects. These toxins appear to act on the plasma membrane, and this is the site at which they initiate the events that culminate in neuromuscular blockade. For additional details, see the paper by Simpson, Lautenslager, Kaiser, and Middlebrook (1993).

ii) MICROSCOPY STUDIES. A postdoctoral associate in my laboratory for six months, Dr. Gabriela Canziani (who left for a postdoctoral position at USAMRIID and is now at FCRDC), devoted most of her time to light and electron microscopy studies on tissues from crotoxin-treated mice. One project was directed toward the visualization of crotoxin on ultrathin sections of intoxicated neuromuscular junctions from systemically

intoxicated mice. We attempted to determine whether there was any evidence of toxin internalization of the neurotoxin. Tissue was removed from animals sacrificed up to two hours following injection of 2 LD_{50}'s of crotoxin, embedded and fixed in the absence of osmium tetroxide, and treated with polyclonal antibodies raised against the basic subunit of crotoxin. This was followed by incubation with a second antirabbit-antibody conjugated to gold spheres. Current methodology appears to be adequate to visualize crotoxin. The amount of labeling observed is quite limited on the neuromuscular junctions isolated from the diaphragm.

Histological and cytological examination of muscle tissues of mice which have an acquired resistance to crotoxin have been carried out in conjunction with Dr. J. C. Vidal (see Okamoto *et al.*, 1993). These studies indicate a dramatic alteration of diaphragm muscle organization in mice treated with low doses of crotoxin, that are now resistant to up to 12 LD_{50}'s of the toxin. Collagen appears to replace 80-90% of the diaphragm muscle tissue. There is evidence of muscle fibre regeneration if crotoxin administration is halted. Dr. Canziani is preparing a manuscript describing these observations, but at this writing I have not yet seen a final version that is ready for submission. An abstract (Canziani et al.,1995) has been published. We could find no previous description in the literature of chronic, systemic intoxication by neurotoxins with PLA_2 activity. At present we have no evidence of crotoxin localization nor internalization.

Dr. Robert Jenkins and his laboratory at the University of Wyoming, have continued these studies in an attempt to get a definitive answer. Two goals were established. One was to track the fate of crotoxin at motor end plates, which required a reliable technique for labeling end-plates. The other was to further improve procedures for labeling crotoxin in diaphragm tissue.

Motor end-plate labeling. Current theories of crotoxin's mode of action, point to its effects on the presynaptic side of motor end-plates. To track crotoxin binding and positively localize it at motor end-plates, a reliable technique for labeling end-plates post-synaptically was developed. Fluorophore-conjugated α-bungarotoxin proved suitable. Previous work showed that α-bungarotoxin binds post-synaptically at acetylcholine channels, and the images gathered on a confocal laser scanning microscope (CLSM) using α-bungarotoxin as a site-selective probe proved effective. Further, a-bungarotoxin binds firmly to tissues and holds up well to a variety of fixation and rinsing procedures.

Tracking of crotoxin in diaphragm tissue. Finding motor end-plates using α-bungarotoxin was quite successful, but finding a technique that clearly labeled crotoxin was more difficult. Indirect immunofluorescence was initially tried, with various times used for tissue fixation. Toxin was applied by either injection of live mice or by soaking excised diaphragm tissues in crotoxin solutions. In all instances, some specific binding of toxin was observed, but non-specific background staining--due either to non-specific trapping of crotoxin in the tissues, non-specific labeling by the primary, secondary, or both antibodies, or a combination of all of these factors--was so high that consistent, reproducible images could not be obtained. Further, specific binding observed was not localized to end plates. In all images, some diffuse staining of nerve bodies was observed.

A second approach consisted of direct conjugation of crotoxin with fluorescein isothiocyanate (FITC). This fluoroprobe is an amine-reactive dye which is widely used in fluorescent microscopy, and reacts readily with primary amines such as the ε-amino group of lysine. By reaction of about 1.5 mole of FITC with 1 mole of Mojave toxin, followed by Sephadex G25 chromatography to separate unreacted FITC from derivatized Mojave toxin, Mojave toxin was isolated that had about one mole of FITC bound per mole. It s toxicity in mice was about the same as unmodified toxin. Preliminary images with this material show Mojave toxin concentrated at the motor end-plate in a small quantity, with a greater quantity binding to neurons leading back to the phrenic nerve. The phrenic nerve itself has the appearance of a braided cable when stained with the FITC-Mojave toxin, perhaps due to staining individual neurons within the nerve. Controls with FITC-labeled BSA indicate that the neural body binding of Mojave toxin is specific.

In other parallel studies, where both FITC-Mojave toxin and labeled bungarotoxin were injected into live mice and their diaphragms viewed immediately after excision, fixing of tissues had a dramatic effect. If fixed, Mojave toxin could not be located; α-bungarotoxin was readily observed. Also, time following injection dramatically affects the observed distribution of Mojave toxin. Studies are continuing through the summer using funds available to Dr. Jenkins and his group.

7. SPIDER VENOM PROTEINS. Several years ago we collaborated with Dr. George Odell at Oklahoma State University on the isolation and characterization of two peptides that were isolated from the venom of the

Mexican red knee tarantula (*Brachypelma smithii*). Preliminary results indicated that one of these peptides had sequence similarities to myotoxin a, a small protein present in venom from *Crotalus viridis viridis*. A brief description of our findings is presented in the paragraph below, which can be read in detail in our paper by Kaiser et al., 1994.

Venom of the Mexican red knee tarantula (*Brachypelma smithii*) was fractionated by gel filtration over Sephadex G-50 Fine. Small polypeptides present in the second and third peaks were subfractionated by cation exchange and reversed-phase FPLC. One major, basic protein was isolated and sequenced from each G-50 fraction using a gas-phase protein sequencer. Primary structures were completed and confirmed using tandem mass spectrometry and carboxypeptidase digestions. Protein 1 contains 39 residues, including six cysteine residues in three disulfide bonds. It is identical to one of the isoforms of ESTX from the venom of the tarantula *Eurypelma californicum* (Savel-Niemann, 1989). *Brachypelma smithii* Protein 5 contains 34 residues, including six cysteine residues in three disulfide bonds. Disulfide bond assignments for both proteins are provided. Protein 5 shows most similarity with Toxin Tx2-9 from the Brazilian 'armed' spider, but only displays 41% sequence identity. Interestingly, the small myotoxins from rattlesnake venom, myotoxin II from the midget faded rattlesnake, *Crotalus viridis concolor* (Bieber et al., 1987); myotoxin 5 from the prairie rattlesnake *Crotalus viridis viridis* (Aird et al., 1991); and crotamine 4 from the South American rattlesnake *Crotalus durissus terrificus* (Smith and Schmidt, 1990), scored 70, 69, and 67, respectively, out of a possible 229 similarity score. Proteins 1 and 5 appear unrelated to each other.

8. We have conducted experiments on the ACTIVATION OF CROTOXIN and porcine pancreatic phospholipase, in our standard phospholipase assay using phosphatidyl choline and with the synthetic substrate 4-nitro-3-octanoyloxybenzoic acid (NOB). With our standard assay, we see the characteristic activation with intact crotoxin, but not with the basic subunit. Porcine pancreatic PLA_2 shows about a 40-fold stimulation following incubation with the substrate for about 20 min. Using 1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine as substrate, with crotoxin, we found at most 1-3% of the molecules monoacylated with the radioactive fatty acid. With porcine pancreatic PLA_2 no label was found to

be incorporated. These values are substantially lower than expected, based on earlier work by Dr. Robert Heinrikson using [^{14}C]NOB. We were unable to obtain any of this labeled substrate from Heinrikson, nor was Dr. Bruce Branchini, from whom we have obtain unlabeled NOB, able to synthesize any of the labeled material. It is not available commercially.

Additional stimulation of PLA_2 activity by transglutaminase was investigated according to the reported procedures from Mukherjee's group (Cordella-Miele et al., 1990; 1993). Pre-incubation of porcine pancreatic PLA_2 with transglutaminase at 37° for 30 min stimulated PLA_2 activity by 100-200% over control pancreatic PLA_2. Similar levels of enzyme activation were observed with intact crotoxin and notexin. When transglutaminase was replace with activated blood clotting factor XIII, no activation was observed. Activity stimulation was observed when transglutaminase (1U/mg protein) was used at a concentration of 0.2 U/ml and porcine pancreatic PLA_2 at a concentration of 5 nM, as described by Mukherjee. Activation was not observed when transglutaminase concentration was decreased 20-fold, with a concomitant increase in notexin to 5 μM. The problem with these experiments, as well as those reported by Mukherjee is that on a weight:weight basis, the transglutaminase is present in nearly a 3,000-fold excess over the PLA_2!

In transglutaminase modifications of both porcine pancreatic PLA_2 and notexin, we find low levels of incorporation of spermidine by transglutaminase. Using 'normal' assay conditions (20 μM substrate, 50 μM ^{14}C-spermidine, 5 μM transglutaminase, and 1 mM DTT), less than 2% of the substrate PLA_2 incorporated spermidine. Dimethylcasein was a much better substrate, and about 40% of the molecules were modified, assuming one mole of spermidine incorporated per mole of casein. We were unable to reconcile Mukherjee's findings with ours, despite extensive laboratory studies.

9. GLYCOPROTEINS. Using an enzyme immunoassay for the detection of carbohydrate in glycoconjugates (from Boehringer-Mannheim), we screened a number of purified presynaptic neurotoxins available to us. Out of these (ammodytoxin, β-bungarotoxin, caudoxin, crotoxin, Mojave toxin, notexin, notexin II-5, pseudexin, textilotoxin, and taipoxin), only textilotoxin and taipoxin contained detectable carbohydrate. Higher molecular weight proteins in crude venoms from *Pseudechis porphyriacus* and *Notechis ater*

occidentalis also gave strong positive signals for the presence of carbohydrates. Based on treatment of O- and N-glycosidases, these higher molecular weight proteins appear to be N-linked and not associated with presynaptic neurotoxins--our main interest.

Taipoxin was shown to be a moderately acidic sialo-glycoprotein (pI 5), heterotrimeric protein with a molecular weight of *ca.* 46 kDa. All of the carbohydrate is contained in the γ-subunit, which includes 4-5 residues of sialic acid, 4-5 residues each of N-acetyl-D-glucosamine and galactose, two residues of mannose, and one residue of fucose (Fohlman et al., 1976). Carbohydrate is N-linked through the Asn-78 in the γ-subunit (Fohlman et al., 1977; Gogain and Kaiser, unpublished results). The γ-subunit could be separated from the α- and β-subunits by gel filtration. Briefly, native taipoxin was dissolved in 6M guanidine-HCl in 50mM MES (pH 6.0) and run over a Superdex 75 HR 10/30 column. SDS-PAGE showed the γ-subunit was largely free of α- and β-subunits. Treatment of the isolated, but not reduced γ-subunit, with the enzyme N-Glycanase gave only partial deglycosylation. For example, when samples were put on a dot-blot and assayed for carbohydrate with the glycan detection kit, deglycosylation appeared almost complete. But when the same material was run on SDS-PAGE and silver stained, low mobility of part of the sample suggested that only partial deglycosylation occurs. To separate deglycosylated γ-subunit from unreacted material, a lectin affinity column was prepared using wheat germ agglutinin covalently bound to 3M Biosupport media AB1. Passage of the partially deglycosylated γ-subunit over the column gave an relatively small unbound fraction and a much larger bound fraction. Unbound material gave a negative carbohydrate test by the dot-blot assay and also migrates faster upon SDS-PAGE and does not stain positively for carbohydrate. One of the technical problems is that the yield of deglycosylated γ-subunit is poor. In reconstitution experiments, recombination of unmodified, isolated γ-subunit with α- and β-subunits gave i.v. LD_{50}-values of ≈3μg/kg in mice, or a toxicity level that was only about 1/3 as toxic as native intact taipoxin. Attempts to reconstitute deglycosylated γ-subunit with α- and β-subunits were unsuccessful, due to sample losses probably resulting from precipitation or adsorption to surfaces. This work was hampered by the lack of adequate amounts of deglycosylated γ-subunit. A better method for the quantitative removal of the sugar moiety from the γ-subunit needs to be developed.

10. PLATELET ACTIVATING FACTOR. We conducted studies to determine whether platelet-activating factor (PAF) is increased in cultured endothelial cells in the presence of presynaptic neurotoxins. B_vV_e endothelial cells (passages 5-9) were utilized for a series of PAF stimulation experiments with thrombin, ionophore A23187, crotoxin and other factors. Procedures described by Prescott et al. (1984) were adapted for use with bovine rather than human cell lines. B_vV_e cells were cultured with endothelial growth media in 25cm^2 flasks to average yields of 7.6 x 10^6 confluent cells/cm^2 surface area. Modifications were made in the cell stimulation and chloroform extraction procedures; including washing cells with Hank's buffered salt solution before treatments with thrombin or ionophore, increasing incubation times, increasing concentrations of thrombin or other factors, pH adjustments, and pre-warming all solutions. Methodology for the cell culturing, chloroform extraction, thin-layer chromatography, and [^{14}C]acetate incorporation were worked out, and essentially followed the published procedures. We observed no stimulation of PAF production in B_vV_e cells treated with crotoxin. However, even with our positive controls we did not observe the type of responses that we would have liked. The results were at best inconclusive. We wanted to examine PAF production in primary cultures of bovine umbilical cords, but the veterinary laboratory with which we were collaborating in collecting tissues had significant bacterial/viral contamination problems during the caving season and was unable to collect usable tissue. Thus, to our chagrin, we discontinued the PAF experiments without obtaining conclusive results.

11. A HETERODIMERIC NEUROTOXIN FROM THE VENOM OF THE FALSE HORNED VIPER (*PSEUDOCERASTES FIELDI*). Heterodimeric neurotoxic proteins have been isolated from some venoms of snakes of the Viperidae family. While a large number of monomeric PLA_2s have been sequenced, the library of sequences for the heterodimeric neurotoxins is limited. Rattlesnake presynaptic neurotoxins consist of an acidic, PLA_2-derived, but enzymatically inactive subunit and a toxic, basic, enzymatically active PLA_2. How the acidic subunit structurally interacts with the basic subunit and increases its toxicity are active areas of research. *Pseudocerastes fieldi* venom contains a two-component neurotoxin which contains a non-toxic, acidic, weakly PLA_2-active component and a toxic, basic PLA_2-active

component. From earlier, unpublished mixing experiments in collaboration with Dr. Avner Bdolah (Tel Aviv University, Israel), we determined that the acidic subunit of *P. fieldi* heterodimer would not potentiate the toxicity of the basic subunit of Mojave toxin. Yet in other ways, the heterodimers of rattlesnake neurotoxins and *P. fieldi* appeared related. This led us to further collaborate with Dr. Bdolah and our eventual decision to sequence both subunits of the heterodimeric neurotoxin of *P. fieldi*, which is summarized below.

The main toxic component of the venom of the false horned viper, *Pseudocerastes fieldi*, is a heterodimeric neurotoxin composed of a basic subunit, Cb II and one of two acidic subunits, Cb Iα or Cb Iß. The nontoxic acidic subunits increase the toxicity of the basic subunit. Both subunits have PLA_2 amino acid sequences. Cb Iα and Cb Iß themselves are inactive towards phosphatidylcholine and when complexed with Cb II promote a delay in the onset of phospholipase activity of Cb II. Cb Iα and Cb Iß do hydrolyze the synthetic substrate, 3-octanoyloxy-4-nitrobenzoic acid, but at <1% the rate of Cb II. High amino acid sequence identity is observed between Cb II and Cb Iα and the basic and acidic subunits respectively of similar heterodimeric neurotoxins. Comparisons of the amino acid sequences of Cb II and Cb Iα with those of other monomeric and dimeric PLA_2s in venoms from Viperidae snakes show that the sequence of the amino acids which are different in the acidic and basic subunits occur in normally highly conserved sequences. This suggests that these amino acid changes in the conserved regions are important for the structure and function of the heterodimeric proteins. Details of this work may be seen in the paper by Francis, Bdolah, and Kaiser (1995a).

12. SUBUNIT ASSOCIATIONS IN PLA_2 PRESYNAPTIC NEUROTOXINS.

Subunit associations of *P. fieldi* neurotoxin and rattlesnake presynaptic neurotoxins, as well as interactions of individual acidic and basic subunits from these heterodimeric complexes have been examined by gel-filtration and light scattering techniques. Substantial differences in elution volumes from gel-filtration columns can result from pH solvent changes, addition of Ca^{++}-ions, and anion differences, suggesting changes in subunit interactions. Light scattering measurements carried out in parallel however, suggest that in most cases elution changes are best explained by altered binding of proteins to the gel filtration matrix and not by changes in

subunit association states. Subunit associations in each toxin have ionic and hydrophobic components. Intact Mojave toxin is highly resistant to trypsin digestion, but the basic subunit by itself is not. Initial cleavage of the basic subunit occurs at the end of the N-terminal helix (Arg-14) suggesting that this region is protected from trypsin digestion by association with the acidic subunit. Using these results, the primary sequence of each toxin's subunit, our knowledge of the structure of the pro-acidic crotoxin subunit, immunological results, and modeling of crotalid PLA_2s--based on the structure of *Crotalus atrox* PLA_2--we have proposed a model for rattlesnake heterodimeric subunit interactions. Surface regions of the acidic and basic subunits likely to be involved in in subunit associations are identified. Since *P. fieldi* toxin basic subunit does not have the same hydrophobic and basic regions as crotoxin basic subunit, and *P. fieldi* toxin acidic subunit does not have the same hydrophobic and acidic regions as crotoxin acidic subunit, the two types of association probably have little in common. However, acidic subunit binding to part of the basic subunit interfacial binding surface may provide a similar mechanism for potentiation of toxicity. This exercise provides a working model for heterodimeric toxin structure.

We presented an abstract on this work at the 5th Pan American Symposium on Animal, Plant and Microbial Toxins in 1995 (*Toxicon* **34**, 286, 1996), and have recently submitted a full-length manuscript (Francis et al., 1996d) entitled "Subunit associations in rattlesnake presynaptic neurotoxins and Pseudocerastes fieldi toxin" to *Toxicon*. A copy of the pre-print has been sent to Ms. Patricia McAllister.

13. CLASSIFICATION OF SNAKE VENOM GROUP II PHOSPHOLIPASES A_2 ACCORDING TO AMINO ACID SEQUENCE. It has often been observed that the amino acid sequence of a PLA_2 or PLA_2-like protein from a particular snake venom has higher amino acid identity with certain PLA_2s from other snake venoms than it does with other PLA_2s from the same snake venom. For example, PLA_2s from the same venom have sometimes been characterized as acidic and basic and the acidic proteins may be more homologous with other acidic PLA_2s and the basic with other basic PLA_2s. These observations suggest that PLA_2s can be classified according to their amino acid sequences. This classification may reflect in-part the genus of the snakes concerned and in-part conserved amino acid sequences. It may also

distinguish between their biological and pharmacological effects. Evidence has been accumulating over many years that toxic PLA_2s exert their toxicity in different ways and their selectivity and potency may be related to initial binding events which occur before PLA_2 activity begins (Rosenberg, 1986). Little information on PLA_2 receptor binding specificity is currently available and a classification scheme based on amino acid sequences might guide future studies on this subject.

During our sequencing of the basic Cb II and acidic Cb Iα subunits of the heterodimeric presynaptic neurotoxin from *Pseudocerastes fieldi* venom, we noticed high conservation of amino acid sequence between Cb II and basic subunits of other heterodimers, and also between Cb Iα and corresponding acidic subunits (Francis et al., 1995a). Apart from amino acids involved in PLA_2 activity, Ca^{2+} binding, disulfide bonds and other residues which are conserved in all group II PLA_2s and appear to fulfill a function in tertiary structure, conservation of sequence is high in other regions. Since certain sequences of the basic, toxic subunits are conserved and variable from the corresponding sequences of the acidic, non-toxic subunits, it is expected that they are involved in the structure and function of the heterodimers. Although a few of the conserved and different amino acids are found in other segments of the subunits, most are bunched in three regions. Using the numbering system of Renetseder et al. (1985) they are: 1) a sequence from 10 to 24 near the N-terminus, 2) a short sequence 34-38, and 3) the C-terminal sequence, 107-133/134. However, the C-terminal regions of the neurotoxic basic subunits such as that from *P. fieldi* show little sequence homology with those of other neurotoxins including the ammodytoxins, caudoxin and the basic subunit of crotoxin. C-terminal sequences found in ammodytoxins, contain amino acids which Gubensek and coworkers have shown to be partly involved in their neurotoxicity
(Ritonja et al., 1986; Krizaj et al., 1989; Curin-Serbec et al., 1991). Amino acids in this region have also been shown to make major contributions to the myotoxicity of *Bothrops asper* K-49 proteins (Lomonte, 1994).
Lysine-38 is a residue implicated in myotoxicity (Francis et al., 1991a) and a preliminary survey indicated that lysine was observed at position 38 in neurotoxic PLA_2s. Since crystal structure determinations show that position 38 is adjacent to the C-terminal sequence in the tertiary structure of PLA_2s (Dijkstra et al., 1983; Brunie et al., 1985; Arni et al., 1995), together they might form a toxic surface region. These observations prompted us to

classify group II PLA_2s on the basis of their C-terminal and 10-24 sequences and then to investigate homology in other segments of the sequences including that containing position 38.

This classification is the basis of a review paper we were invited to write as a chapter in the book entitled *The Enzymology of Snake Venoms*, edited by G. S. Bailey, which should be published in late 1996 or early 1997 by Alaken, Inc. Press (305 W. Magnolia St., Suite 196, Fort Collins, CO 80521). The review is divided into seven segments, which are titled as follows: (1) Classification of group II PLA_2s, (2) Correlation of subgroups with biological activities, (3) Division of PLA_2 sequences into 13 segments, (4) Specificity of biological activity, (5) Amino acids involved in myotoxicity and neurotoxicity, (6) Heterodimeric PLA_2 associations, and (7) PLA_2 aggregation. It is cited in the LITERATURE CITED section as Francis, Meng, and Kaiser (1996c).

A copy of the classification review was sent to my Technical Representative (c/o Ms. Patricia M. McAllister, USAMRAA, SGRD-RMA-RC, Ft. Detrick, Frederick, MD 21702-5014) in May, 1996. If additional copies are needed, please let me know.

b. Molecular Biology.

1. GENOMIC CLONES. When this work was initiated, there had been a number of reports in the literature of PLA_2 cDNA clone isolation, but few reports describing the isolation of their genomic clones and none describing the isolation of genomic PLA_2 clones from snakes. As a first step in better understanding the evolution of presynaptic neurotoxins and gaining some insight into genomic control regions, we initiated the isolation and nucleotide sequencing of the genomic clones encoding both the non-neurotoxic, non-enzymatic acidic subunit and the toxic, PLA_2-active basic subunit of Mojave toxin.

Mojave toxin is a heterodimeric, neurotoxic PLA_2 found in the venom of the Mojave rattlesnake, *Crotalus scutulatus scutulatus,* and is characteristic of all rattlesnake presynaptic neurotoxins. Work which we completed and published (John, Smith, and Kaiser, 1994) describes the isolation and nucleotide sequence of the genomic clones encoding both the non-neurotoxic, non-enzymatic acidic subunit (*mtx-a*) and the toxic, PLA_2-

active basic subunit (*mtx-b*), and compares their structures. We demonstrated that both genes shared virtually identical overall organization, with four exons separated by three introns, which were inserted in the same relative positions of the genes' coding regions. The exon/intron structure was similar to that reported for mammalian PLA_2 genes. Most remarkable was the high degree of nucleotide sequence identity between *mtx-a* and *mtx-b*. While the exons shared about 70% identity, the introns were greater than 90% identical and the 5' and 3' untranslated and flanking regions were greater than 95% identical. These findings support our earlier suggestion (Aird, Kaiser, Lewis, and Kruggel, 1985) that the genes coding for the two subunits arose from a common ancestor. There has clearly been a strong selection on the nucleotide sequence of the non-coding regions during this evolutionary process. This is the first report of genomic sequences of PLA_2-like proteins from snakes. Additional details of this study may be found in the paper by John et al., 1994.

2. PSEUDO-GENES. During our work involving the sequencing of the genes for Mojave toxin acidic and basic subunits described above, we noted that the coding regions shared about 70% sequence identity, while the introns and untranslated and flanking regions were >90% identical. This permitted us to propose a generalized structure for group II PLA_2 genes (see Fig. 11 in APPENDIX). The first exon codes for a signal peptide which is post-cleaved to form the mature peptide while exons 2, 3, and 4 code for the structural protein. Remarkably, the genes for Mojave toxin and Habu snake PLA_2 isoforms (Nakashima *et al.*, 1993; 1995) share highly similar nucleotide sequences, where the non-coding DNA is more similar to the corresponding region of the PLA_2 gene than is the coding DNA. Recently, Moura-da-Silva *et al.* (1995) isolated a PLA_2 cDNA clone from the viperid snake *Bothrops jararacussu* and compared its nucleotide sequence to PLA_2 cDNA from *C. d. terrificus* and *Trimeresurus flavoviridis* noting the higher similarity of 5' and 3' untranslated regions with respect to coding regions between these species. Non-coding regions of this *Bothrops* PLA_2 cDNA also share high sequence identity to corresponding regions of the acidic and basic subunit genes of Mojave toxin from *C. s. scutulatus* and pgPLA 1a, pgPLA 1b, and BP-I from *T. flavoviridis*. In this extended study on the genome of the Mojave rattlesnake, we present the nucleotide sequence for a PLA_2-like pseudogene. Except for lacking the first exon found in other snake Group II PLA_2 genes,

Pseudo-Mojave toxin gene exhibits the same gene structure and sequence similarities found in the genes for the acidic and basic subunits of Mojave toxin, as well as the Habu snake PLA_2 isoform genes.

The phospholipase A_2 pseudogene we discovered, isolated, and characterized is located 2000 nucleotides upstream, and on the opposite DNA strand, from a gene for Mojave toxin acidic subunit. The pseudogene lacks the first exon and a few segments of non-coding DNA found in functional snake venom PLA_2 genes, but does have the coding information for a complete PLA_2 protein. Pseudogene retains the unusual gene sequence similarity pattern found in functional viperid PLA_2 genes. When compared to genes from *C. s. scutulatus* and the Habu snake (*Trimeresurus flavoviridis*), pseudogene shows strong conservation of non-coding regions and variable protein-coding regions. While the non-coding regions of pseudogene are conserved with respect to other viperid PLA_2 genes, the three exons code for a unique PLA_2-like protein most closely related to ammodytoxin b found in the venom of the western sand viper (*Vipera ammodytes ammodytes*). The structure of these genes suggests a common ancestor for all viperid PLA_2 genes. For details and a phylogenetic analysis of our investigations into the genomic structure of the Mojave rattlesnake, see John, Smith, and Kaiser (1996).

A reviewer of this paper indicated that he agreed with our assessment, that "these genes provided a textbook example of molecular evolution". He went on to say that he felt "these early papers on venom phospholipase A_2 evolution will turn out to be **classics**".

3. EXPRESSION OF THE BASIC AND ACIDIC SUBUNITS OF MOJAVE TOXIN. Early in this work we constructed an *E. coli* expression vector (pTM-N) which contained the *ompA* signal peptide (21 amino acids) and a linker peptide (6 amino acids) connected to the amino-terminal end of the basic subunit protein, giving a primary product 149 amino acids long. The linker peptide contained the tetra peptide Ile-Glu-Gly-Arg, immediately ahead of the basic subunit protein's N-terminal His, which is recognized by the proteinase Factor X_a. The vector is transcribed by T7 polymerase, which is present in the host bacterial chromosome under *lac* UV5 control and inducible by addition of IPTG. SDS-PAGE gels and western blots of cell extracts from induced cultures, indicated that 3-4% of the total cellular protein was represented by the basic subunit protein (122 amino acids),

containing the 27 amino acid fusion peptide at the amino-terminal end, for a product of 149 amino acids in length (data not shown).

Model studies on the reduction and re-oxidation of the basic subunit of Mojave toxin (see section b. 4 below), indicated that we could reconstitute 40-45% of the PLA_2 activity of the reduced, purified basic subunit. Using protein product recovered from our expression system and the same reduction and re-oxidation conditions, we were unable to generate any significant phospholipase activity. We attributed this to (i) the hydrophobic *ompA* fusion peptide, which promoted solubility problems, and (ii) our inability to easily purify the expressed product, to eliminate contaminating and possibly interfering proteins. Because of this we designed and constructed seven additional expression plasmids; four containing the basic subunit cDNA and three containing the cDNA for the proacidic subunit. Most were designed to promote solubility of the product and facilitate its isolation and purification. I will describe the construction for one of these in some detail. The others were constructed in a similar manner. All are based on the pET-vector series and are listed in Table 1 in the APPENDIX.

Expression vector pET 19b (Novagen, Madison, WI), contains a leader peptide coding for a polyhistidine region (Met-Gly-His_{10}) followed by the underlined enterokinase cleavage site (Ser-Ser-Gly-His-Ile-Asp-Asp-Asp-Asp-Lys), immediately on the amino-side of the N-terminal His of the basic subunit protein. The polyhistidine and enterokinase regions should provide a hydophilic tail, improving the solubility of our expressed fusion protein product. In addition, the polyhistidine region can be used as a handle to affinity purify the product on a Ni-column. Construction of the vector and cDNA are outlined in Fig. 12. As with the pTM vector, the pET-vector series is also transcribed by T7 RNA polymerase which is constructed into the host cell chromosome under *lacUV* control and can be induced by IPTG.

Transformed BL21 (no T7 pol gene, consequently a non-expressing host strain) and BL21(DE3) (contains T7 pol gene) cells, containing our newly constructed expression plasmid grew well in L-broth. When IPTG was added to both cultures at mid-log phase, there was no evidence of a newly synthesized protein in the 18 kD molecular weight region on SDS-PAGE in BL21 cells, but a prominent band was present in the BL21(DE3) cells as shown in Fig. 13A. Western blots using polyclonal antibodies raised to Mojave toxin basic subunit also gave a strong signal in the 18 KD region from extracts from BL21(DE3) cells (see Fig. 13B). Sequencing of the expression

vector containing the cDNA insert, identified as pTJ7-133#a16, gave the expected sequence with no deletions at the ligation junction sites that would alter the reading frame of the cloned cDNA.

A construct similar to this was prepared using the cDNA for the pro-acidic subunit of Mojave toxin. When expressed in BL21(DE3) cells, a new protein of the appropriate molecular weight was found in the cell extract as determined by SDS-PAGE. Western blots using rabbit polyclonal raised to the acidic subunit, but not the basic subunit, gave a strong signal at the expected position (Fig. 14). Isolation and subsequent re-oxidation of the expressed protein containing either the basic or acidic subunit were unsuccessful.

Two additional expression vectors (using Novagen's pET-17xb), one with the proacidic subunit cDNA and one with the basic subunit cDNA were constructed. In both constructs, an N-terminal leader of the first 265 amino acids of the T7 phage gene 10 protein, was followed by the five amino acids which make up the enterokinase proteolytic site, was followed by the 122 amino acids of the appropriate subunit protein. We were hopeful that the long leader in the fusion product would facilitate solubilization of the expressed subunit proteins. Induction in host strain BL21(DE3)pLysE showed good expression when the extract in run on SDS-PAGE and western blots using the homologous polyclonal antibody raised to either the basic or acidic subunit gives a strong signal (data not shown). Thus, we are overexpressing both subunits. Isolation and reoxidation of the expressed proteins did not yield phospholipase-active proteins

Three additional expression plasmid constructions were prepared, which consisted solely of either the acidic or basic subunit cDNAs (lacking the native Mojave toxin signal peptide) preceded by the translational start codon ATG. Evidence suggested that upon recombinant protein translation, endogenous host *E. coli* methionine amino peptidase will cleave the N-terminal Met residues, leaving native basic subunit or (pro)acidic subunit. This cleavage appears favored when the amino acids on the carboxyl-side of Met are small or uncharged amino acids. In our new Met-Mojave toxin constructions, the penultimate amino acid residue for the acidic subunit is Ser, and for the basic subunit His. In theory, Ser should promote cleavage and His should not. Thus, we designed to constructs for the basic subunit, one of which has the N-terminal Met followed by the native His residue and one where the His is replaced by Ser. These constructs have been transferred into various *E. coli* expression strains and induced. The basic subunit-

containing strains express well, as determined by SDS-PAGE and protein staining as well as by western blots (data not shown). The acidic subunit-containing strains appear to express much more poorly, but show a strong positive signal on western blots when reacted with a rabbit polyclonal primary antibody. Once again, isolation and oxidation of these expressed protein products did not yield phospholipase-active proteins. All expression clones prepared, as well as the site-directed mutants of Mojave toxin basic subunit are listed in Table 1 in the APPENDIX.

4. RENATURATION OF EXPRESSED PROTEINS. One of the technical difficulties of expressing eukaryotic proteins in a prokaryotic system, is proper folding of the protein and proper reoxidation of disulfide bonds. To this end, we have conducted a number of model studies on the reduction and re-oxidation of the basic subunit of Mojave toxin, using published reoxidation procedures. In our hands, we found that with a buffer consisting of 50mM Tris-HCl (pH 8-8.5), 10-15mM cysteine, 10mM $CaCl_2$, and 0.2-0.25% Triton X-100, we could reconstitute 40-45% of the PLA_2 activity of the reduced basic subunit over a 48-72 hour period. We tried several other published renaturation conditions with the reduced basic subunit, but none of them were as efficient as the one described. When the above reconstitution system was used in an attempt to generate phospholipase activity in protein product recovered from our *E. coli* expression systems, we were unable to generate <u>any</u> enzymatic activity. Repeated efforts using a variety of renaturation techniques were also unsuccessful. We feel that our inability to renature these expressed subunits are in-part, the result of their intrinsicly low solubilities, which leads to their precipitation from solution. This appears to be exacerbated by the high cysteine content of these subunits, which appear to form intermolecular disulfide bonds resulting in insoluble, higher molecular weight aggregates. This is suggested by extraction experiments on expressed basic subunit containing the poly-histidine at the N-terminal end. For example, this product could not be extracted from the pellet with either 6M urea, 6M guanidine hydrochloride, EDTA, or DTT alone, but could be extracted with a combination of DTT and urea. Affinity purified (Ni-column) product also appears aggregated. Triton X-100 solubilized the expressed product, but does not completely disperse the aggregates.

Efforts to express these proteins as 'fused proteins' using leaders with a high degree of hydophilicity to make them soluble were also unsuccessful.

As a result of our inability to renature the expressed proteins, we finally decided to put this problem on the shelf until something 'new' in the renaturation area was available to try. We felt we had pretty well exhausted the renaturation procedures published to date. During these studies, we did demonstrate that Factor X_a activity is preserved in the presence of 0.2-0.25% Triton X-100 as long as 100mM NaCl is present.

5. MUTAGENESIS STUDIES. A major goal of our laboratory has been to better understand the nature of toxicity of snake venom PLA_2 neurotoxins. While our renaturation efforts on the expressed Mojave toxin subunits were still in progress, we decided to explore a site-directed mutagenesis system for introducing amino acid changes at selected sites in the cloned, basic subunit. Using Promega's 'Altered Sites II' site-directed mutagenesis kit, we prepared two mutations in the basic subunit. In one, Lys-38 was converted to Gln-38; and in the other, Tyr-115 was converted to Asp-115 [using the Renetseder et al.,(1985) numbering system]. This mutagenesis system appears to work well and indicates that future site-specific mutagenesis with these subunits should not present a problem--once a procedure for the renaturation of the expressed subunits is developed. See Table 1 for additional details on these mutants.

c. Immunology.

1. ANTIGENICITY OF POST-SYNAPTIC NEUROTOXINS. During our purification of proteins from *Notechis scutatus scutatus* using cation-exchange chromatography, we identified several basic proteins with apparent molecular weights of ≈11 kD. We have demonstrated that one of these proteins is notechis III-4, a "long" postsynaptic neurotoxin containing 73 amino acids (Halpert et al., 1979). Polyclonal antibodies raised against notechis III-4 has been used to investigate the presence of similar postsynaptic toxins in *N. s. scutatus* venom and the venoms of other snakes.

Our (Francis, Tanaka, and Kaiser, 1993a) demonstrates that polyclonal antibodies raised against purified notechis III-4, a postsynaptic neurotoxin from the Australian tiger snake, *N. s. scutatus*, recognizes conformational and linear epitopes in notechis III-4, but only conformational epitopes in other *N. s. scutatus* venom proteins including notexin and notechis II-5. Notechis III-4 is markedly deficient in venom from *N. s. scutatus* collected

near Lake Alexandrina in Australia, and absent from venoms of *Notechis ater* subspecies, crotalids, and viperids. Of six other elapid venoms screened, only *Bungarus multicinctus* and *Pseudonaja textilis* showed weak cross-reactivity. Reactive protein species in these venoms include the long post-synaptic neurotoxins α-bungarotoxin and pseudonajatoxin b. Surprisingly the polyclonal antibodies were only weakly neutralizing against notechis III-4 lethality. These studies indicate that the most antigenic regions of the long postsynaptic toxins are the variable or non-conserved regions. A new method for purification of notechis III-4 is reported.

2. TOXIC SITES OF RATTLESNAKE PRESYNAPTIC NEUROTOXINS. Based in-part on some of our earlier unpublished work involving the Geysen procedure and studies reported on ammodytoxin by F. Gubensek (Curin-Serbec et al., 1991), we had five peptides synthesized corresponding to different regions of crotoxin/Mojave toxin. These peptides corresponded to different regions of the basic subunit and are identified as follows: K1 = amino acids 1-9; K2 = 31-44; K3 = 90-103; K4 = 102-114, and K5 = 113-122 (see Fig. 15 for the Mojave toxin basic subunit sequence). Each peptide was attached to the new multiple antigenic peptide system (MAPS), which utilize the α- and ε-amino functional groups of lysine to form a backbone to which multiple peptide chains can be bound. We used an eight peptide branch core to which identical peptides were attached, giving a synthetic system purported to be highly antigenic. Two rabbits were immunized with each peptide using Titermax adjuvant, followed by four boosts. Our results were disappointing and are briefly summarized below.

i) When reacted with homologous antigen, anti-K3 reacts strongly (1000-fold dilution); anti-K2 and K5 reacts moderately (10-50-fold dilution); anti-K1 and K4 were very weak (<10-fold dilution).

ii) Antisera generated against intact crotoxin as well as the individual subunits of crotoxin did not react with any bound K1-K4 peptides. Peptide K5 was recognized moderately by antisera against intact crotoxin as well as monoclonal antibodies 2, 1, and 5. However, when peptide K5 was pre-incubated with either the polyclonal or monoclonal antibodies prior to mixing with crotoxin, their ability to inhibit the toxin's PLA_2 activity or lethality was not affected.

iii) Antisera raised in only one of two rabbits immunized with K5 reacted with either basic subunit or intact crotoxin (250-fold dilution). This

antisera had no affect on crotoxin's PLA_2 activity or lethality. Antisera from none of the other 9 rabbits reacted with intact crotoxin.

We were disappointed that peptide conjugation to the 'multiple antigenic peptide system' and then used to immunize rabbits with Titermax adjuvant, yielded such poor antibody response. The major experimental differences between our protocol and that of Gubensek's (Curin-Serbec et al., 1991), was that his group used keyhole limpet hemocyanin as a carrier protein and Freund's adjuvant during immunization of rabbits. Gubensek had success with this same basic approach using peptides derived from the sequence of ammodytoxin.

As a result of our negative results, we essentially repeated our experiment using Gubensek's protocol as closely as possible. We purified and conjugated the five peptides corresponding to the same five regions of the basic subunit of Mojave toxin as noted above, to keyhole limpet hemocyanin. Each of these five conjugated peptides were used to immunize two rabbits each, using Gubensek's protocol, which involved an initial injection using complete Freund's adjuvant and five boosts using incomplete Freund's adjuvant. Characterization of the antisera gave the following results. (i) Antisera raised against a conjugated peptide, reacted specifically with that peptide only, showing no cross-reactivity with the other four peptides. (ii) When KLH-peptide was bound to an ELISA plate and assayed, the KLH-peptide reacted most strongly with its homologous antisera. All antisera recognized KLH carrier itself. (iii) Two of the antisera showed high titers against the basic subunit and intact form of crotoxin, but no reactivity toward the acidic subunit. (iv) Phospholipase assays with intact crotoxin and the basic subunit, with various antisera suggest that there is only slight (up to 35%) inhibition of enzymatic activity. (v) None of the sera, or purified IgG recovered from the sera by protein A purification, showed any indication of toxin neutralization of either Mojave toxin or crotoxin when premixed and injected either i.p. or i.v. in mice.

These findings and others, are substantially different from those reported by Gubensek (Curin-Serbec et al., 1991), on peptide antisera prepared against sequences from the related ammodytoxins. We have critically compared our results with his, and have been unable to satisfactorily reconcile the differences.

3. ANTIGENICITY STUDIES OF NON-TOXIC AND ENZYMATICALLY INACTIVE MOJAVE TOXIN SUBUNITS EXPRESSED IN E. COLI. In studies done in collaboration with Drs. N. Nascimento and Rogero (Sao Paulo, Brazil), we demonstrated that γ-radiation of crotoxin led to decreased toxicity, but retention of antigenicity. For details see paper by Nascimento et al. (1996). This suggested that even though the basic subunit and pro-acidic form of the acidic subunit of Mojave toxin that were expressed in our *E. coli* system were enzymatically inactive and non-toxic, they may serve as useful antigens to generate antisera effective in neutralizing native crotoxin. To examine these possibilities we expressed two different basic subunit constructions, referred to as Met-MTX-b (pTJ 10-79#17) and Met-Ser-MTX-b (pTJ 10-79#28), and one acidic subunit construction, referred to as Met-MTX-a (pTJ 10-79#1), in *E. coli* expression strain BL21. See Section 3 under MOLECULAR BIOLOGY and Table 1 in APPENDIX.

After preparation of the cell-free extracts of the reduced forms of all three expressed proteins, they were each fractionated by gel filtration (Superdex 75) in 6M guanidine-HCl/50 mM Tris-HCl (pH 8). The appropriate peaks were pooled, dialyzed and lyophilized. SDS-PAGE of the recovered fractions showed the recovered, recombinant proteins were >90% pure and they electrophoresed at the expected molecular weight. N-terminal sequencing of these recovered proteins showed the following:

Protein	Sequence Found	Expected
Met-MTX-b	Met-His-Leu-Leu...	Met-His-Leu-Leu...
Met-Ser-MTX-b	Ser-Leu-Leu-Gln...	Met-Ser-Leu-Leu-Gln... (and/or) Ser-Leu-Leu-Gln...
Met-MTX-a	A 1:1 mix of the following N-termini: Met-Ser-Leu-Val-Glu... Ser-Leu-Val-Glu-Phe...	Met-Ser-Leu-Val-Glu... (and/or) Ser-Leu-Val-Glu...

Two rabbits were immunized with each antigen (200μg intradermally, and boosted with 100μg i.m. 3x). Sera antibody titers were determined by ELISAs on plates coated with various antigens.

Antisera raised against Met-MTX-b reacted weakly (10-50x dilution of antisera) with intact Mojave toxin, reduced basic subunit, and control basic subunit. It does not react with control or reduced acidic subunit from Mojave toxin.

Antisera raised against Met-Ser-MTX-b reacted weakly (10-250x dilution of antisera) with reduced, intact Mojave toxin and reduced basic subunit, as well as control basic subunit. It does not react with either control acidic subunit or reduced acidic subunit.

Antisera raised against Met-MTX-a reacted strongly against its homologous antigen (32,000x dilution) and more weakly against control and reduced acidic subunit (250-1,250x dilution). It did not cross-react with control or reduced basic subunit.

Because of the weak titer the antisera raised against the basic subunit construct proteins showed, work with these antisera was discontinued. We suspect that the low solubility of the basic subunit construct proteins is responsible for their low antigenicity.

Antisera raised against Met-MTX-a was screened for its ability to neutralize intact crotoxin. One ml of antisera with the highest titer available was mixed with 200μl of PBS containing 10μg/ml of intact Mojave toxin. Incubation was for 1 hr at 37^{o} before injection. At a dose of 2x the LD_{50}-value in male mice (0.1μg/g), all animals challenged died between 2-3 hrs. Similar results were obtained with preimmunization sera. We conclude that the antisera raised against Met-MTX-a is not effective in neutralizing the toxicity of intact Mojave toxin.

4. AN ANTI-CROTOXIN COMBINATORIAL ANTIBODY SELECTED FROM A PHAGE-DISPLAYED LIBRARY. Immune passive therapy is the treatment of choice for snake bite victims. This practice requires production of a high-titer antisera from animals, which may be difficult to generate with some snake venoms containing high concentrations of potent neurotoxins. In these circumstances antisera spiked with neutralizing monoclonal antibodies can be used to enhance the neutralizing ability of antivenoms (Kaiser and Middlebrook, 1988a). While effective under laboratory conditions, production of large amounts of monoclonal antibodies for therapeutical use is not presently economically feasible. Combinatorial antibody generation using molecular cloning techniques is an alternative to the production of monoclonal antibodies using traditional hybridoma technology.

We describe in this work the utility of this approach in the preparation of a crotoxin-specific, high-affinity, single-chain Fv (scFv) monoclonal antibody generated by combinatorial methods using Pharmacia's Recombinant Phage Antibody System. A high affinity clone, designated A10G, was selected and its DNA sequence determined. Protein A10G showed high

reaction specificity, with only the closely related rattlesnake neurotoxins, concolor toxin and Mojave toxin showing cross-reactivity out of eleven group II PLA_2s screened. No group I PLA_2s cross-reacted in enzyme-linked immunosorbent assays. The gene coding for A10G was subcloned into an expression vector and the resulting expressed nonfusion protein, designated A10GPE, was renatured and purified to apparent homogeneity. Dissociation constants of A10G with intact crotoxin, and crotoxin basic subunit were determined to be 7×10^{-10} M and 6.8×10^{-9} M, respectively. When A10GPE was pre-incubated with either the basic subunit or intact crotoxin at molar ratios of up to 5:1, no inhibition of phospholipase activity was observed. Expressed protein could however, partially neutralize the lethality of the crotoxin homolog, Mojave toxin, in mice. Additional details on this work may be found in the paper by Meng, John, and Kaiser (1995).

d. Exploratory Studies.

1. ENDOGENOUS TOXIN INHIBITORS. Snakes protect themselves from the effects of their own toxin venom components in a variety of ways. Some snake sera, and selected mammalian sera, contain glycoproteins that complex with venom hemorrhagic proteinases and thereby neutralize their activity. This work has been expanded in our Antiproteolytic/Antihemorrhagic experiments described in section (ii) below. Cholinergic receptors isolated from the cobra (*Naja naja atra*), are resistant to binding α-bungarotoxin, a postsynaptic neurotoxin from *Bungarus multicinctus*, a related elapid, even though they readily bind both acetylcholine and d-tubocurarine, explaining why elapids are unaffected by their own postsynaptic neurotoxins (Ohana et al., 1991). In related work, described in section (iii) below, we were unable to detect the presence of any postsynaptic neurotoxin inhibitors present in elapid snake sera. Venoms of *Bothrops neuweidii* and *Naja naja naja* are reported to contain protein inhibitors of phospholipases (Vidal and Stoppani, 1971; Braganca et al., 1970), although we have been unable to duplicate the result for *N. n. naja*. Many venoms contain high concentrations of citrate that inhibit enzymes, including phospholipases, by metal-ion chelation, as was demonstrated by George Odell's laboratory and ours (Freitas, Geno, Sumner, Cooke, Hudiburg, Ownby, Kaiser, and Odell, 1992; Francis, Seebart, and Kaiser, 1992). Some snake venoms contain small peptides which also act as inhibitors of venom proteinases. Experiments describing the inhibition of

toxic metalloproteinases in *Bothrops asper* venom by endogenous peptides are discussed in the next paragraph.

(i) NATURAL METALLOPROTEINASE INHIBITORS. During our earlier work on the isolation of *Bothrops asper* phospholipase A_2s, we observed that these venoms also have metal-ion dependent proteases and high concentrations of low molecular weight peptides. *In vitro* experiments demonstrated that two of these peptides, pyroglutamate-glutamine-tryptophan (pEQW) and pyroglutamate-asparagine-tryptophan, are present in crude venom at concentrations of about 4.5 and 1 mM, respectively. Proteinase fractions from *B. asper* are inhibited from digesting oxidized insulin B-chain *in vitro* by both of these tripeptides with an IC_{50} for pEQW of ≈0.3 mM. Digestion of purified myotoxin MIII from *B. asper* venom is also inhibited *in vitro* by pEQW, suggesting that similar inhibition of proteinase activities probably occurs in the venom gland. Inhibitory peptides present in venom allow snakes to be protected from their own toxic proteinases and inhibit hydrolysis of venom proteins during storage in the venom gland. Upon dilution, such as when venom is injected into prey, peptide inhibitors dissociate from the proteinase and allow their activation. A manuscript describing these results (Francis and Kaiser, 1993) has been published in *Toxicon.*

This paper is of interest for several reasons. First, it demonstrates a function for the relatively high concentrations of peptides found in *B. asper* venom. It also describes another mechanism by which snakes can protect themselves from their own toxic proteins during storage, and suggests how these naturally occurring peptides may be useful in preparing more potent inhibitors of proteinase activity which might be useful for snake bite therapy. Finally, the results described above, may also be applicable in the clinical treatment of tetanus and botulism. If the recent report by Schiavo et al. (1992) proves correct and tetanus and botulinum toxins are zinc endoproteinases and this activity is essential for intoxication, then inhibitors based on the tripeptide structures we isolated should be screened for their inhibitory properties of these neurotoxins.

(ii) ANTIPROTEOLYTIC/ANTIHEMORRHAGIC SERA PROTEINS. An undergraduate in my laboratory isolated an ANTIPROTEOLYTIC, ANTIHEMORRHAGIC PROTEIN from the sera of the the Western Diamondback rattlesnake (*Crotalus atrox*). Using a combination of $(NH_4)_2SO_4$ precipitation, S-200 gel-filtration, and ion-exchange chromatography, he

isolated a single chain, 58 kD glycoprotein with an estimated pI-value of about 5.4. Treatment with N-glycosidase F and O-glycosidase results in a lower molecular weight band on SDS-PAGE, but no loss of activity. Activity of the separated fractions was monitored by the inhibition of crude *C. atrox* venom proteinase activity on hide powder azure, in lieu of a live animal assay. Purified inhibitor protein protects mice against the hemorrhagic effects of crude *C. atrox* venom on subcutaneous skin tests. When combined with crude venoms, the glycoprotein forms visible complexes on non-denaturing polyacrylamide gels with four different species of crotalids. Fibrinogen hydrolysis by purified hemorrhagic toxin A from *C. atrox*, and a purified hemorrhagin from *C. v. viridis* is also inhibited by the antiproteinase. The first 21 amino-terminal amino acids were determined and shown to be FQLAG NMDVN TKGTK DWADI G... Data base searches indicate that this sequence has no identity with albumins, α-globulins, or macroglobulins, but does show a partial identity with an antihemorrhagic factor isolated from the sera of the Japanese Habu snake.

Natural resistance of some animals to venomous snakebite is not attributable to immunoglobulin s, consistent with the results of this study. Table 1 shows that isolated antihemorrhagic factors are soluble, acidic blood proteins (likely glycoproteins) with molecular weights in the albumin or a-globulin region (except for the hedgehog macroglobulin). They do not form precipitin lines with the venom in immunodiffusion tests, and none have shown proteolytic activity as a means of neutralizing venom toxins. Therefore, it has been suggested that the antihemorrhagic factors form stable, non-covalent, stoichiometric complexes with venom hemorrhagins and proteinases that inhibit their enzymatic activity until the complex can be cleared from the bloodstream.

A manuscript describing the isolation and partial characterization of our protein has been accepted for publication, but with revisions that require additional laboratory work. We have already devoted considerable time and effort to this problem, which has been 'on the shelf' for a couple of years. It does not seem worth-while at this point to initiate new studies on this problem. It may be possible to re-write the manuscript into a short communication and resubmit what we have without additional laboratory studies. I will discuss our options with the co-author Keith Spencer upon his return, who is currently in full-time training with the National Guard.

(iii) We screened several snake sera for the presence of possible

postsynaptic neurotoxin inhibitors. These sera were freeze dried samples supplied by Mr. Peter Mirtschin of Venom Supplies in South Australia. They were reconstituted in our laboratory and assayed for their ability to neutralize the toxicity of notechis III-4, a postsynaptic neurotoxin from the venom of *Notechis scutatus scutatus* venom in mice. Table 2 in the APPENDIX indicates that there is no evidence that any of the four different sera screened (*Notechis scutatus, Pseudonaja textilis, Acanthophis antarcticus, and Oxyuranus microlepidotus*) were able to neutralize the toxin. We observed *O. microlepidotus* sera in one injection to neutralize, but could not repeat the result suggesting that there was a problem with the experiment itself.

2. ANOMALOUS GEL FILTRATION BEHAVIOR. Based on their molecular weights, notexin and scutoxin elute later than expected from gel filtration columns in multiple peaks (See Francis *et al.*, 1991b). Notexins present in these peaks have identical amino acid sequences and unmodified amino acid side chains. Scutoxin is an isoform of notexin which contains arginine at position 16 and glutamate at position 82. Like notexin, it also elutes in several different fractions on a gel filtration column, yet the peaks show identical amino acid sequence. This perplexing chromatographic behavior appears to be caused by the association of these proteins with different anions, since dissolving notexin in buffers containing different anions produces up to a 30% change in elution volume. Certain anions promote an apparent reduction in the interaction of notexin with the gel filtration matrix, hence earlier elution. These anions include citrate, 3-phosphoglycerate and 2-phosphoglycerate, which also inhibit the PLA_2 activity of notexin. However, even under conditions which minimize protein-matrix interaction the toxins elute later than expected based on their molecular weight.

The observed elution volume differences seen with different 'forms' of notexin and scutoxin probably reflect the overall net charge on the proteins that are moderated by anion binding. This overall net charge appears to alter the interaction of the proteins with the gel filtration matrix. Further, anion binding to particular basic amino acids may alter specific interactions with the matrix. For example, guanidino groups bind sulfate and phosphate tightly under physiological conditions (Tatham et al., 1983) and may be expected to bind to other anions. One arginine in notexin, Arg-17, binds sulfate in the

notexin crystal structure but several other arginines do not. Consequently, a number of factors may be involved in determining whether or not anions bind to particular basic amino acids. We reported these results in preliminary form at a meeting of the American Chemical Society (Francis et al., 1995d) and later in a full-length manuscript in *Toxicon* (Francis et al., 1995c).

3. PHOTOAFFINITY LABELING OF COMMON TIGER SNAKE (*Notechis scutatus scutatus*) VENOM PROTEINS WITH [γ-^{32}P]8-AZIDOADENOSINE TRIPHOSPHATE. Snake venom toxicities are clearly related to their protein and peptide content, which represents the major percentage of the venom solids (Stocker, 1990). Lower molecular weight non-protein, organic components found in venoms include lipids, carbohydrates, organic acids, nucleosides, nucleotides, amino acids, biogenic amines, and salts (Stocker, 1990). Functions of these lower molecular weight materials are largely unknown, although the relatively high levels of citrate found in some venoms relative to Ca^{2+}-ion concentrations suggest that they may chelate endogenous divalent metal ions, thereby inactivating divalent cation-requiring enzymes. ATP, which is also found in some venoms at mM concentrations (Francis et al., 1992) may also serve as a divalent-metal chelator. We were interested in examining whether there were any ATP-specific binding proteins present in Tiger snake (*Notechis scutatus scutatus*) venom which we showed earlier to have ATP concentrations approaching 10mM. We carried out photoaffinity labeling of *N. s. scutatus* venom using γ-labeled 8-azidoadenosine triphosphate [γ-^{32}P]8N_3ATP. This azido analog was originally synthesized in Haley's laboratory (Potter and Haley, 1983), and over the years has proven to be an invaluable tool in identifying ATP-binding proteins and more recently in characterizing ATP-binding sites. With many ATP-binding proteins the 8N_3-analog mimicks ATP, except that it is particularly sensitive to ultraviolet light. When irradiated with short wavelength ultraviolet light, the azido group decomposes to N_2 and a nitrene -- which can undergo a number of reactions, including the formation of covalent bonds between the analog and amino acid side-chains. Thus, when proteins are mixed with [γ-^{32}P]8N_3ATP at the appropriate concentration and irradiated with ultraviolet light, the radioactive nucleotide becomes photoincorporated into the binding proteins. Such photolabeled proteins can then be fractionated and their properties studied.

Crude, dialyzed *N. s. scutatus* venom was incubated with increasing

concentrations of [γ-^{32}P]8N_3ATP, photolyzed, and analyzed by SDS-PAGE. A typical gel stained with coomassie is shown as an insert in Fig. 16. When three different regions of the gel were excised as shown in the insert in Fig. 16, and counted for radioactivity, the results shown in Fig. 16 were obtained. Increasing concentrations of the probe resulted in increasing photoincorporation, with an indication of saturation, particularly in band 4 near 1000 μM 8N_3ATP. This suggests that the protein(s) in band 4 was specifically labeled. From Fig. 16, we assumed an apparent K_d of band 4 protein(s) to be $\approx$400μM. By plotting the radioactivity recovered in different regions of the gel on top of the scanned Coomassie stained bands, multiple experiments gave high specific activities for band 4 (Fig. 16). This high specific activity further suggested specific binding of the nucleotide to the protein(s) in band 4. Further, if the probe is mimicking ATP and labeling is at the active site of an enzyme specific for ATP, then by increasing the concentration of ATP in the photolabeling experiment should decrease the extent of incorporation. By the same reasoning, photoincorporation should not be greatly affected by the presence of an unrelated nucleotide which does not compete for the binding site.

When [γ-^{32}P]8N_3ATP concentration is held constant with increasing concentrations of cold ATP, we found band 4 decreased with increasing ATP concentration as shown in Table 3, Expt. 1. In this band, ATP appears to have a lower affinity for the binding site (higher K_d-value) than does 8N_3ATP. If the labeling is specific for ATP then other nucleotides should have little affect on photoincorporation. In one experiment, using a 1-minute incubation of the venom with the natural nucleotide, followed by the addition of [γ-^{32}P]8N_3ATP, 1.2mM ATP reduced the label incorporated into band 4 by 31%; ADP by 9%; and CTP by 19% (see Expt. 2, Table 3). In an independent experiment (Expt. 3, Table 3), where 1.2mM ATP decreased photoincorporation by 38%, 1.2mM ADP and CDP decreased the labeling 26 and 16%, respectively. While the purine diphosphate was a less effective competitor than ATP, it was still competitive. Pyrimidine nucleotides were generally less effective. This indicates that both the phosphate residues and base moieties of the triphosphates are involved in the nucleotide-protein interactions, and that the photoincorporation of 8N_3ATP is not absolutely ATP-specific. This perhaps is not too surprising if one considers that many of the known potential nucleotide-binding proteins present in venoms such as the 5'-

nucleotidases, phosphatases, phosphodiesterases, and nucleases are not base specific. An ATPase has been reported (Iwanaga and Suzuki, 1979), but we did not observe what could be considered an ATP-specific binding protein in *N. s. scutatus* venom. Thus, these photolabeling experiments were unable to provide insight into why some venoms contain high concentrations of endogenous ATP.

In the absence of any photolysis, we found no evidence of any labeled proteins present in *N. s. scutatus* venom following incubation with [γ-^{32}P]8N_3ATP and that the radioactive label results from photoincorporation of the nucleotide and not phosphorylation. We are preparing a short communication of this work for submission (Kaiser and Ray, 1996).

CONCLUSIONS

Phospholipase A_2s isolated from snake venoms have generally been described as neurotoxic, myotoxic, cardiotoxic or non-toxic as demonstrated by toxicity assays conducted by injections in mice. PLA_2s which are non-toxic may exhibit a biological activity, such as platelet aggregation or hemolysis, but this activity may not be lethal to mice. We have identified a new type of toxic PLA_2 that is present in some elapid snake venoms. After injection of these toxins i.v. in mice, hemorrhage is observed in several tissues, but is most evident in the lungs where blood is observed in the thoracic cavity. After i.p. injection hemorrhage is observed in the peritoneal cavity. In cases studied to date, a rapid loss of blood pressure is also observed. Based on these pharmacological activities, we have called these proteins hypotensive/hemorrhagic (HT) PLA_2s.

These HT PLA_2s are promising models to understand certain aspects of septic shock syndrome, which is characterized by refractory hypotension and progressive organ damage leading to death, a condition often accompanied by hemorrhage.

Preliminary results obtained with Dr. A. Bieber on the N.M.R. structural analysis of a purified isoform of the acidic subunit of Mojave toxin (which we provided to his laboratory) are promising and are continuing. Attempts by Dr. David Scott to prepare crystals of Mojave toxin (using highly purified toxin provided by our laboratory), suitable for heavy-atom replacement x-ray analysis studies have been unsuccessful to date. It may be that the multiple isoforms of the toxin naturally present in samples of Mojave toxin prevent

growth of crystals of adequate quality.

Work on the enzyme 'destabilase' from leech saliva has been discontinued since a satisfactory source of leech saliva proved to be unavailable. We directed our attention to a chemically cleavable cross-linker EGS and have successfully cross-linked intact crotoxin, as evidenced by SDS-PAGE, ion-exchange chromatography, and gel-filtration. We have demonstrated that cross-linked crotoxin can be cleaved with hydroxyl amine and both the cross-linked and cleaved crotoxin retains phospholipase activity. Characterization is continuing. This groundwork should permit an answer of 'whether the two subunits of rattlesnake presynaptic neurotoxins need to separate to be toxic'.

Collaborative experiments in conjunction with Dr. Lance Simpson's laboratory, indicate that PLA_2 neurotoxins are not endocytosed to exert their effects. PLA_2 neurotoxins appear to bind to the cell surface and then undergo a molecular rearrangement that gives them access to critical substrates in the membrane which leads to a blockade of neuromuscular transmission.

Microscopy studies carried out in parallel with the pharmacology experiments, have proven to be extremely difficult. Methodologies that work successfully for localizing α-bungarotoxin do not work with the rattlesnake presynaptic neurotoxins. A number of techniques have been improved upon, but little in the way of definitive results have been achieved.

Two major peptides from the venom of the Mexican red knee tarantula (*Brachypelma smithii*) were isolated, characterized by sequencing, and analyzed with the existing protein sequence database. Small myotoxins from rattlesnake venoms appear only distantly related.

We find little (1-3% of molecules) acylation of crotoxin following incubation with 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine substrate. This is substantially less acylation than reported earlier by Heinrikson's laboratory. We were unable to determine the disparity between our results and his. In transglutamination experiments, following the procedures of Mukherjee, minimal amounts of activation of neurotoxic PLA_2s were observed. We found no evidence that transglutamination plays any role in stimulating presynaptic neurotoxins.

Of ten PLA_2 presynaptic neurotoxins examined, we found only textilotoxin and taipoxin contained detectable carbohydrate. The γ-subunit of taipoxin and D-subunit of textilotoxin are the glycosylated moieties. Taipoxin was shown to be a moderately acidic sialo-glycoprotein (pI$\approx$5),

heterotrimeric proteins with a molecular weight of ca. 46 kDa. All of the carbohydrate is contained in the γ-subunit, which includes 4-5 residues of sialic acid, 4-5 residues each of N-acetyl-D-glucosamine and galactose, two residues of mannose, and one residue of fucose. Carbohydrate is N-linked through the Asn-78 of the γ-subunit. Gamma-subunit could be separated from the α- and β-subunits by gel filtration. Treatment of native γ-subunit with N-glycanase gave only partial deglycosylation. Totally deglycosylated material could be separated from the remaining material by chromatography over a lectin affinity column. Yields of deglycosylated material were poor and were the limiting factor in conducting additional experiments. A better method for the quantitative removal of the sugar moiety from the γ-subunit need to be developed.

A system for examining the effects of various factors on platelet activating factor (PAF) generation in cultured endothelial cells was established. We observed no stimulation of PAF production in bovine B_vV_e cells treated with crotoxin under our conditions.

Comparisons of the amino acid sequences of Cb II and Cb Iα from the two-component neurotoxin from *P. fieldi* venom with those of other monomeric and dimeric PLA_2s in venoms from Viperidae snakes show that the sequence of the amino acids which are different in the acidic and basic subunits occur in normally highly conserved sequences. This suggests that amino acid changes in the conserved regions are important for the structure and function of the heterodimeric proteins.

Subunit associations of *P. fieldi* neurotoxin and rattlesnake presynaptic neurotoxins, as well as interactions of individual acidic and basic subunits from these heterodimeric complexes have been examined by gel-filtration and light scattering techniques. Based on experiments in a variety of buffer conditions, we conclude that subunit associations in each toxin have both ionic and hydrophobic components. Using proteolytic hydrolysis experiments, the primary sequence of each toxin's subunit, our knowledge of the structure of the pro-acidic crotoxin subunit, immunological results, and modeling of crotalid PLA_2s--based on the structure of *C. atrox* PLA_2--we have proposed a model for rattlesnake heterodimeric subunit interaction. Surface regions of the acidic and basic subunits likely to be involved in subunit associations are identified.

PLA_2s from the same venom have sometimes been characterized as acidic and basic, and the acidic proteins may be more homologous with other

acidic PLA_2s and the basic with other basic PLA_2s than with each other. These observations suggest that PLA_2s can be classified according to their amino acid sequences. This classification may reflect in-part the genus of the snakes concerned and in-part conserved amino acid sequences. We have proposed a new classification system for group II PLA_2s, based on their C-terminal and 10-24 sequences, and then compare homology in other segments of the sequences, including position 38.

When this experimental work was initiated, there had been a number of reports in the literate of PLA_2 cDNA clone isolation, but few reports describing the isolation of their genomic clones and none describing the isolation of genomic PLA_2 clones from snakes. We described the isolation and nucleotide sequence of the genomic clones encoding both the non-neurotoxic, non-enzymatic acidic subunit and the toxic, PLA_2-active basic subunit, and compared their structures. Both genes shared virtually identical overall organization with four exons separated by three introns, which were inserted in the same relative positions of the genes' coding regions. The exon/intron structure was similar to that reported for mammalian PLA_2 genes. Most remarkable was the high degree of nucleotide sequence identity between the two subunit genes. While the exons shared about 70% identity, the introns were greater than 90% identical and the 5' and 3' untranslated and flanking regions were greater than 95% identical. These findings support our earlier suggestion that the genes coding for the two subunits arose from a common ancestor.

The phospholipase A_2 pseudogene we discovered, isolated, and characterized is located 2000 nucleotides upstream, and on the opposite DNA strand, from a gene for Mojave toxin acidic subunit. The pseudogene lacks the first exon and a few segments of non-coding DNA found in functional snake venom PLA_2 genes, but does have the coding information for a complete PLA_2 protein. Pseudogene retains the unusual gene sequence similarity pattern found in functional viperid PLA_2 genes. When compared to genes from *C. s. scutulatus* and the Habu snake (*Trimeresurus flavoviridis*), pseudogene shows strong conservation of non-coding regions and variable protein-coding regions. While the non-coding regions of pseudogene are conserved with respect to other viperid PLA_2 genes, the three exons code for a unique PLA_2-like protein most closely related to ammodytoxin b found in the venom of the western sand viper (*Vipera ammodytes ammodytes*). The structure of these

genes suggests a common ancestor for all viperid PLA_2 genes.

We have constructed plasmid expression vectors for both the acidic and basic subunits of Mojave toxin using their cDNAs. SDS-PAGE and western blots indicate good expression of both subunit proteins in *E. coli* following induction in a variety of expression vectors. Isolation and subsequent re-oxidation of the expressed protein containing either the basic or acidic subunit were unsuccessful.

We conducted a number of model studies on the reduction and re-oxidation of the basic subunit of Mojave toxin. One of these allowed us to reconstitute 40-45% of the PLA_2 activity of the reduced basic subunit over a 48-72 hour period. When the above reconstitution system was used in an attempt to generate phospholipase activity in protein product recovered from our *E. coli* expression systems, we were unable to generate <u>any</u> enzymatic activity. Repeated efforts using a variety of renaturation techniques were also unsuccessful. We feel that our inability to renature these expressed subunits are in-part, the result of their intrinsicly low solubilities, which leads to their precipitation from solution. This appears to be exacerbated by the high cysteine content of these subunit, which appear to form intermolecular disulfide bonds resulting in insoluble, higher molecular weight aggregates.

A mutagenesis system which we employed, appears to work well and indicates that future site-specific mutagenesis with these subunits should not present a problem--once a procedure for the renaturation of the expressed subunits is developed.

Immunology studies with the "long" postsynaptic neurotoxin notechis III-4 from *N. s. scutatus* indicate that most antigenic regions of these neurotoxins are the variable or non-conserved regions. Polyclonal antibodies raised to the protein appear to be weakly neutralizing and show some cross-reactivity with conformational epitopes in the presynaptic neurotoxins of notexin and notechis II-5.

Attempts to raise polyclonal antibodies to peptides from various regions of the basic subunit of Mojave toxin were disappointing. We have used three different carrier proteins, but peptides conjugated to any of these carriers have not generated useful antibodies. These findings and others, are substantially different from those reported by Gubensek, on peptide antisera prepared against sequences from the related ammodytoxins. We have critically compared our results with his, and have been unable to

satisfactorily reconcile the differences.

Antisera raised against Met-MTX-b expressed in *E. coli* and purified reacted weakly (10-50x dilution of antisera) with intact Mojave toxin, reduced basic subunit, and control basic subunit. It does not react with control or reduced acidic subunit from Mojave toxin. Antisera raised against Met-MTX-a expressed in *E. coli* and purified reacted strongly against its homologous antigen (32,000x dilution) and more weakly against control and reduced acidic subunit (250-1,250x dilution). It also does not cross-react with control or reduced basic subunit. Based on these results, we conclude that the antisera raised against either Met-MTX-a or -b, are not effective in neutralizing the toxicity of intact Mojave toxin.

We described the preparation of a crotoxin-specific, high-affinity, single-chain Fv (scFv) monoclonal antibody generated by combinatorial methods using Pharmacia's Recombinant Phage Antibody System. A high affinity clone, designated A10G, was selected and its DNA sequence determined. Protein A10G showed high reaction specificity, with only the closely related rattlesnake neurotoxins, concolor toxin and Mojave toxin showing cross-reactivity out of eleven group II PLA_2s screened. No group I PLA_2s cross-reacted in enzyme-linked immunosorbent assays. Despite tight binding, with dissociation constants of $\approx 10^{-10}$M, when A10GPE was pre-incubated with either the basic subunit or intact crotoxin at molar ratios of up to 5:1, no inhibition of phospholipase activity was observed. Expressed protein could however, partially neutralize the lethality of the crotoxin homolog, Mojave toxin, in mice.

Snakes protect themselves from the effects of their own toxic venom components in a variety of ways. Some snake sera, and selected mammalian sera, contain glycoproteins that complex with venom hemorrhagic proteinases and thereby neutralize their activity. This work has been expanded and we have described and partially characterized a antiproteolytic/antihemorrhagic factor present in sera of *C. atrox.* Some snake venoms contain small peptides (pyroGlu-Gln-Trp and pyroGlu-Asn-Trp) which also act as inhibitors of venom proteinases. We have reported experiments in which endogenous peptides in the venom of *B. asper* are potent inhibitors of toxic metalloproteinases found in the same and other venoms. These peptides may have inhibitory activities toward the toxic bacterial zinc proteinases (tetanus and botulinum toxins). In contrast with these positive results, we found no evidence for the

presence of any postsynaptic neurotoxin inhibitors present in elapid snake sera.

We observed anomalous gel-filtration behavior of some elapid PLA_2s. This perplexing chromatographic behavior appears to be caused by the association of these proteins with different anions, since dissolving notexin in buffers containing different anions produces up to a 30% change in elution volume. Certain anions promote an apparent reduction in the interaction of notexin with the gel filtration matrix, hence earlier elution. However, even under conditions which minimize protein-matrix interaction the toxins elute later than expected based on their molecular weight. The elution volume differences seen probably reflect the overall net charge on the proteins that are moderated by anion binding. This overall net charge appears to alter the interaction of the proteins with the gel filtration matrix. Further, anion binding to particular basic amino acids may alter specific interactions with the matrix. A number of factors may be involved in determining whether or not anions bind to particular basic amino acids.

We observed that some snake venoms, such as that from *N. s. scutatus*, contain mM levels of ATP. We examined whether there were any ATP-specific binding proteins present in Tiger snake venom using γ-labeled 8-azidoadenosine triphosphate. It has been successfully used to identify ATP-binding proteins, since it mimics the natural analog and upon photolysis with ultraviolet light can form covalent bonds with the binding protein. In our studies we did not observe what could be considered an ATP-specific binding protein in *N. s. scutatus* venom, and are unable to provide insight into why some venoms contain high concentration of endogenous ATP.

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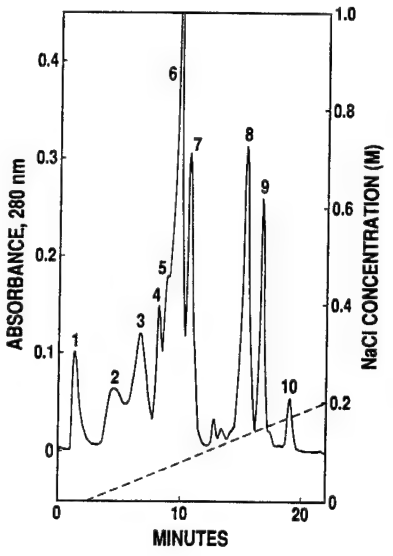
APPENDIX



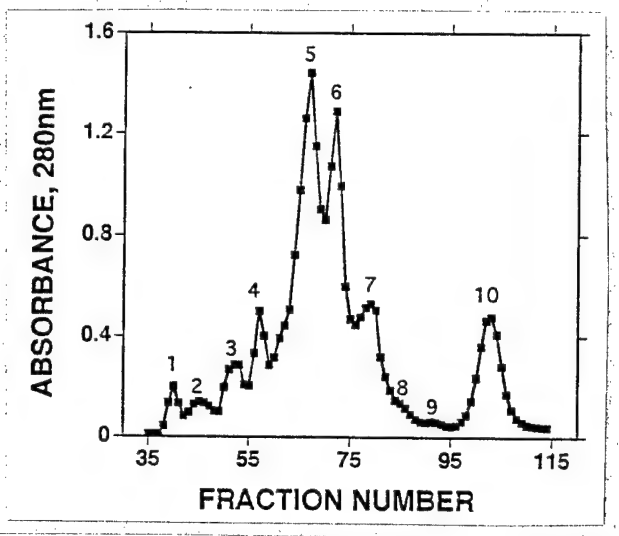

```
                                    25                                  50
1.Notexin Np        NLVQFSYLIQCANHGKRPTWHYMDY GCYCGAGGSGTPVDELDRCCKIHDD
2.Notechis II-5     NLVQFSYLIQCANHGRRPTRHYMDY GCYCGWGGSGTPVDELDRCCKIHDD
3.Notechis 11'2     NLYQFGNMIQCANHGRRPTLAYADY GCYCGAGGSGTPVDELDRCCKAHDD
4.Notechis II-1     NLVQFSNMIQCANHGSRPSLAYADY GCYCSAGGSGTPVDELDRCCKTHDD
5.OS1               SLLNFANLIECANHGTRSALAYADY GCYCGKGGRGTPLDDLDRCCHVHDD
6.B.fasc. PLA2      NLFQFKNMIQCA--GTRSWTDYVSY GCYCGKGGSGTPVDQLLRCCKVHDD
7.Textilotox.D      NIMLFGNMIQCTIPCEQSWLGYLDY GCYCGSGSSGIPVDDVDKCCKTHDE
8.Taipoxin γ        DFEQFSNMIQCTIPCGSECLAYMDY GCYCGPGGSGTPIDDLDRCCKTHDE
9.Porc. pancr.      ALWQFRSMIKCAIPGSHPLMDFNNY GCYCGLGGSGTPVDELDRCCETHDN
10.HTe              NLYQFGNMIQCANHGRRPTRHYMDY GCYCGKGGSGTPVDELDRCCQTHDD

                                    75                                 100
1.Notexin Np        CYDEAGKK-GC     FPKMSAYDY YCGENGPYCRNIKKKCLRFVCDCDV
2.Notechis II-5     CYSDAEKK-GC     SPKMSAYDY YCGENGPYCRNIKKKCLRFVCDCDV
3.Notechis 11'2     CYGEAGKK-GC     YPTLTLYSW QCIEKTPTCNSKTG-CERSVCDCDA
4.Notechis II-1     CYARATKSYSC     TPYWTLYSW QCIEKTPTCDSKTG-CQRFVCDCDA
5.OS1               CYGEAEKLPACNYLMSSPYFNSYSY KCNEGKVTCTDDNDECKAFICNCDR
6.B.fasc. PLA2      CYGDAEKIPKC     KPYYKTYSY DCSEGKLTCKADNDECAAFICNCDR
7.Textilotox.D      CYYKAGQIPGCSVQPNEVFNVDYSY ECNEGQLTCNESNNECEMAVCNCDR
8.Taipoxin γ        CYAEAGKLSACKSVLSEPNNDTYSY ECNEGQLTCNDDNDECKAFICNCDR
9.Porc. pancr.      CYRDAKNLDSCKFLVDNPYTESYSY SCSNTEITCNSKNNACEAFICNCDR
10.HTe              CYGEAEKLPACNYMMSGPYYNTYSY ECNEGELTCKDNNDECKAFICNCDR
11.HTg                    LPACNYMMSGPYYNTYSY ECNEGELTCKDDNNECGAFIC

                                   125
1.Notexin Np        EAAFCFAKA-P-YNNANWNIDTKKR CQ
2.Notechis II-5     EAAFCFAKA-P-YNNANWNIDTKKR CQ
3.Notechis 11'2     TAAKCFAKA-P-YNKKNYNIDTEKR CQ
4.Notechis II-1     TAAKCFAKA-P-YNKENYNIDPKKR CQ
5.OS1               TAAICFAGA-T-YNDENFMISKKYN DICQ
6.B.fasc. PLA2      VAAICFAGA-P-YNDNNFMIDSKTR CQ
7.Textilotox.D      AAAICFARF-P-YNKNYWSINTEIH CR
8.Taipoxin γ        TAVTCFAGA-P-YNDDLYNIGMIE- CHK
9.Porc.pancr.       NAAICFSKA-P-YNKEHKNLDTKKY C
10.HTe              TAAICFARA-P-YNDANWNIDTKTR CQ
```

Fig. 1. Crude *N. s. scutatus* venom was initially fractionated by gel filtration on Sephacryl S200 (Francis et al., 1991b). Acidic proteins present in peak IIB (1.3 mg), unbound at pH 5.5 on Mono S, were applied to a Mono Q column in 50 mM Tris-HCl (pH 7.5). Bound material was eluted with an increasing concentration of 1 M NaCl buffered with 50 mM Tris-HCl (pH 7.5). Protein present in peaks 1-10 were collected and compared by SDS-PAGE.

Fig. 2. Comparison of the amino acid sequences of *N. s. scutatus* HT_e with proteins having related sequences. Numbering system is based on the alignment according to x-ray structure (Renetseder et al., 1985). References for sequences 1 through 9 are Halpert and Eaker (1975), Halpert and Eaker (1976); Bouchier et al. (1991), Lind and Eaker (1980), Lambeau et al. (1995), Liu et al. (1989), Pearson et al. (1991), Fohlman et al. (1977), Puijk et al. (1977), respectively. Textilotoxin D and taipoxin-γ are missing N-terminal residues 1-8.



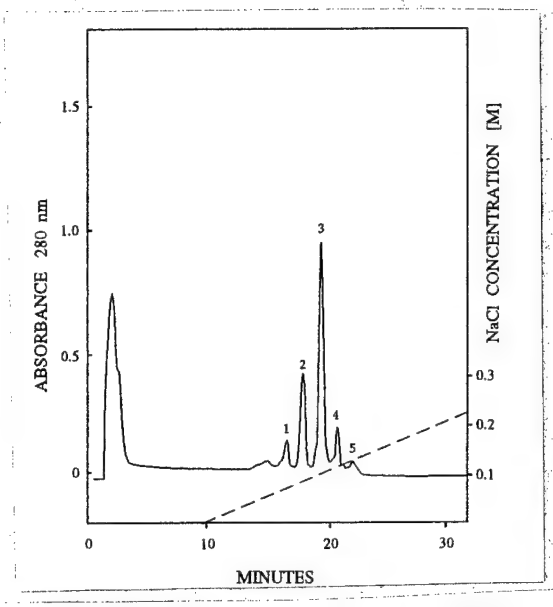
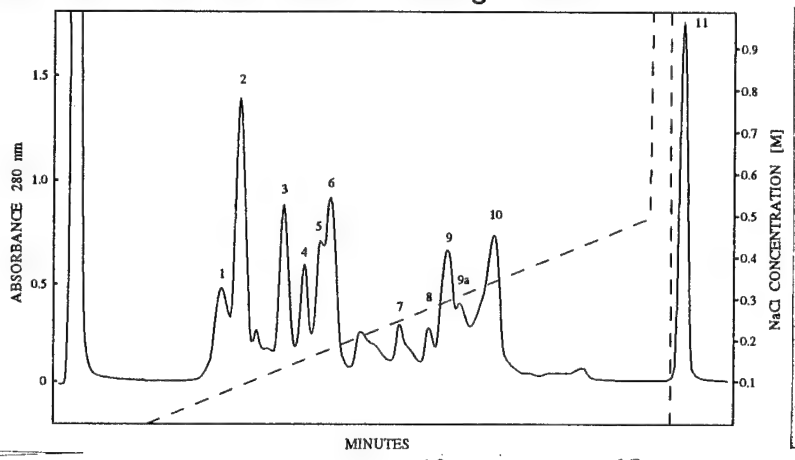


Fig. 3. S200 gel filtration fractionation of crude *M. f. frontalis* venom. Absorbance at 280nm of fractions (23 ml) obtained after applying 0.4 g. *M. f. frontalis* venom dissolved in 10 ml 0.1M sodium acetate, pH 4.5, to a S200-HR gel filtration comumn (5 x 95 cm) equilibrated in the same buffer.

Fig. 4. Mono Q anion exchange chromatography of material in S200 peak 5. Absorbance at 280nm of S200 peak 5 material (2 mg) dissolved in 50mM Tris-HCl (pH 8.5), and applied to a Mono Q column equilibrated in the same buffer. Bound material was collected by elution with an increasing concentration of the same buffer containing 1M NaCl.



	5							10							15							20			
CONSERVED			C			N				S	S					T					C				
PEAK 3	M	I	C	Y	N	H	Q	S	S	E	P	P	T	T	K	T	C	-	-	S					

	25							30							35							40			
CONSERVED	E			C	Y		K		W		D		H				R	G	T						
PEAK 3	E	G	Q	C	Y	K	K	S	X	S	D	-	H	-	-	-	R	G	T	I	I				

Fig. 5A. Mono S cation exchange chromatography of material in S200 peak 5. Absorbance at 280nm of S200 material (8 mg) dissolved in 50mM sodium phosphate (pH 5.0) and applied to a Mono S column equilibrated in the same buffer. Bound material was collected by elutin with an increasing concentration of the same buffer containing 1M NaCl.

Fig. 5B. N-terminal sequence of the first 35 amino acid residues of the postsynaptic neurotoxin isolated from *M. f. frontalis*. Its sequence has been aligned with invariant residues found among short, postsynaptic neurotoxins as illustrated by Endo and Tamiya (1991). (-) indicates inserted gaps and (x) is an unidentified residue.

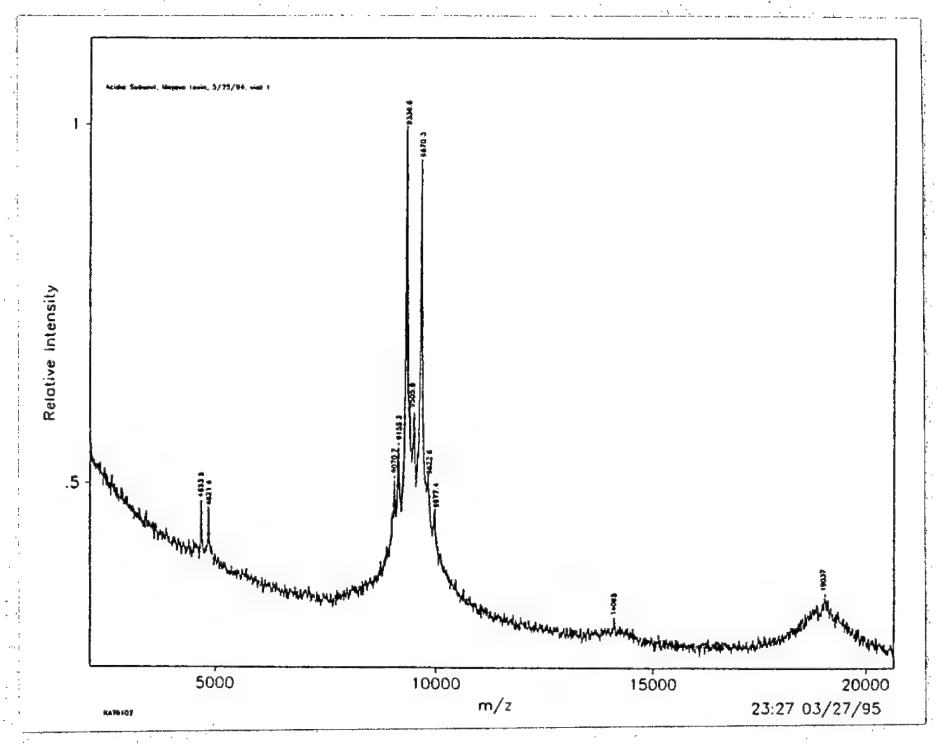


Fig. 6. Typical mass spectrum of the crude, acidic subunit of Mojave toxin, showing multiple isoforms.

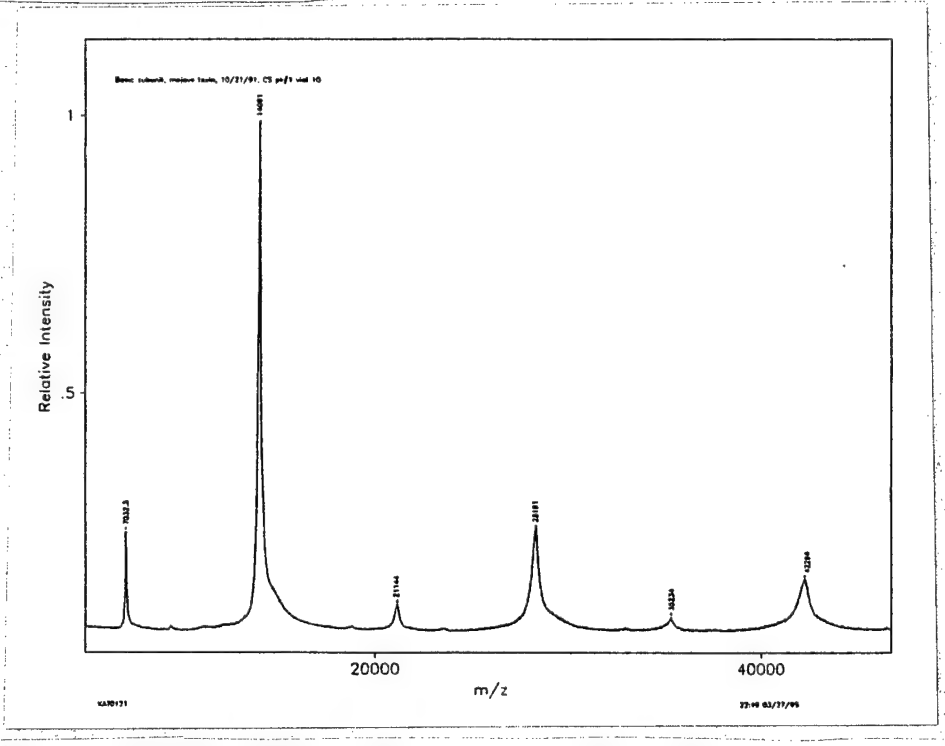


Fig. 7. Typical mass spectrum of crude, basic subunit of Mojave toxin.

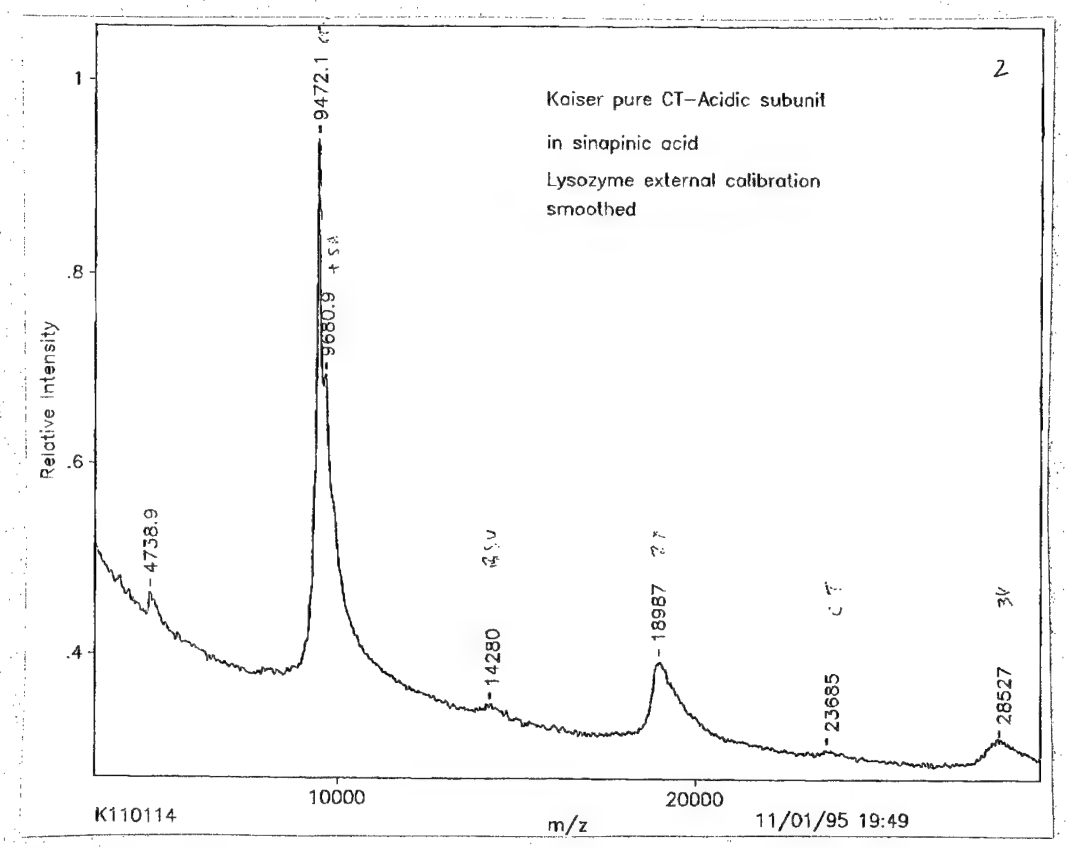


Fig. 8. Typical mass spectrum of a 'highly enriched' acidic subunit isoform of crotoxin after extensive anion exchange and reversed-phase chromatography. We were forced to change to the acidic subunit of crotoxin after crude Mojave rattlesnake venom (*Crotalus scutulatus scutulatus*) was no longer commercially available.

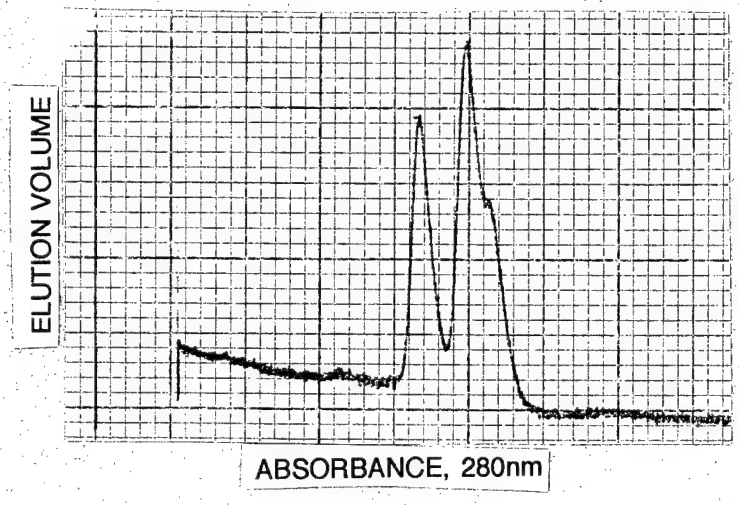


Fig. 9. Superdex 75 gel-filtration profile of cross-linked crotoxin recovered from the initial Mono Q chromatographic run. Samples (200µl) were dissolved in 50mM Tris-HCl (pH 7.6)-6M GuHCl and applied to the 1 x 30 cm Superdex 75 column at room temperature and eluted with the above buffer at 0.3 ml/min and monitored at 280nm.

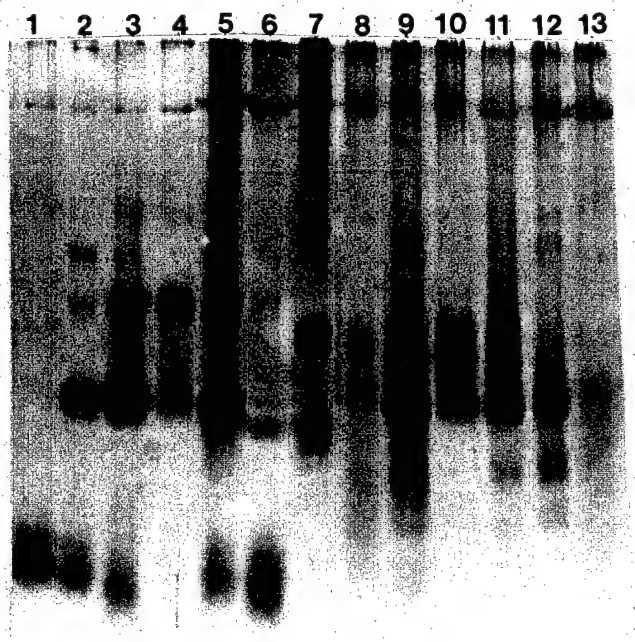


Fig. 10. Various control and cross-linked samples were run on SDS-PAGE (15% polyacrylamide gel) and silver stained. Samples run are as follows: lane 1, unmodified acidic subunit; lane 2, unmodified intact crotoxin; lane 3, cross-linked crotoxin recovered from the Mono Q column; lane 4, cross-linked crotoxin recovered from the Superdex 75 column; lane 5, cross-linked material in lane 4 treated with hydroxylamine; lane 6, modified acidic subunit of crotoxin; lane 7, molecular weight standards with arrows on the right indicating their mobilities. Samples applied to lanes 8-13 were treated with protein solubilizing solution containing DTT before electrophoresis. Lane 8, modified acidic subunit of crotoxin; lane 9, cross-linked material in lane 4 treated with hydroxylamine; lane 10, cross-linked crotoxin recovered from the Superdex 75 column; lane 11, cross-linked crotoxin recovered from the Mono Q column; lane 12, unmodified intact crotoxin; lane 13, unmodified acidic subunit of crotoxin.

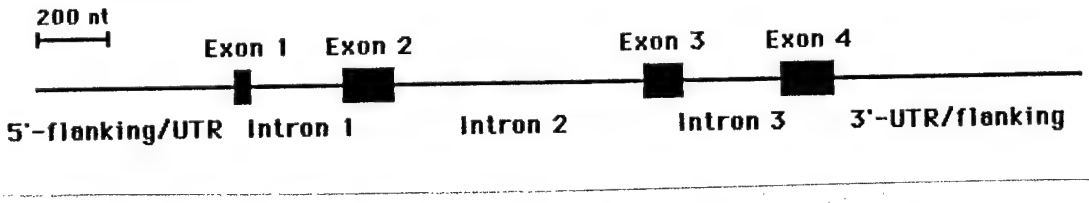


Fig. 11. General structure of snake venom group II PLA2 genes. Coding regions are indicated by boxes and are numbered above the figure; non-coding and flanking regions are indicated by lines between and surrounding the boxes and are identified below the figure.

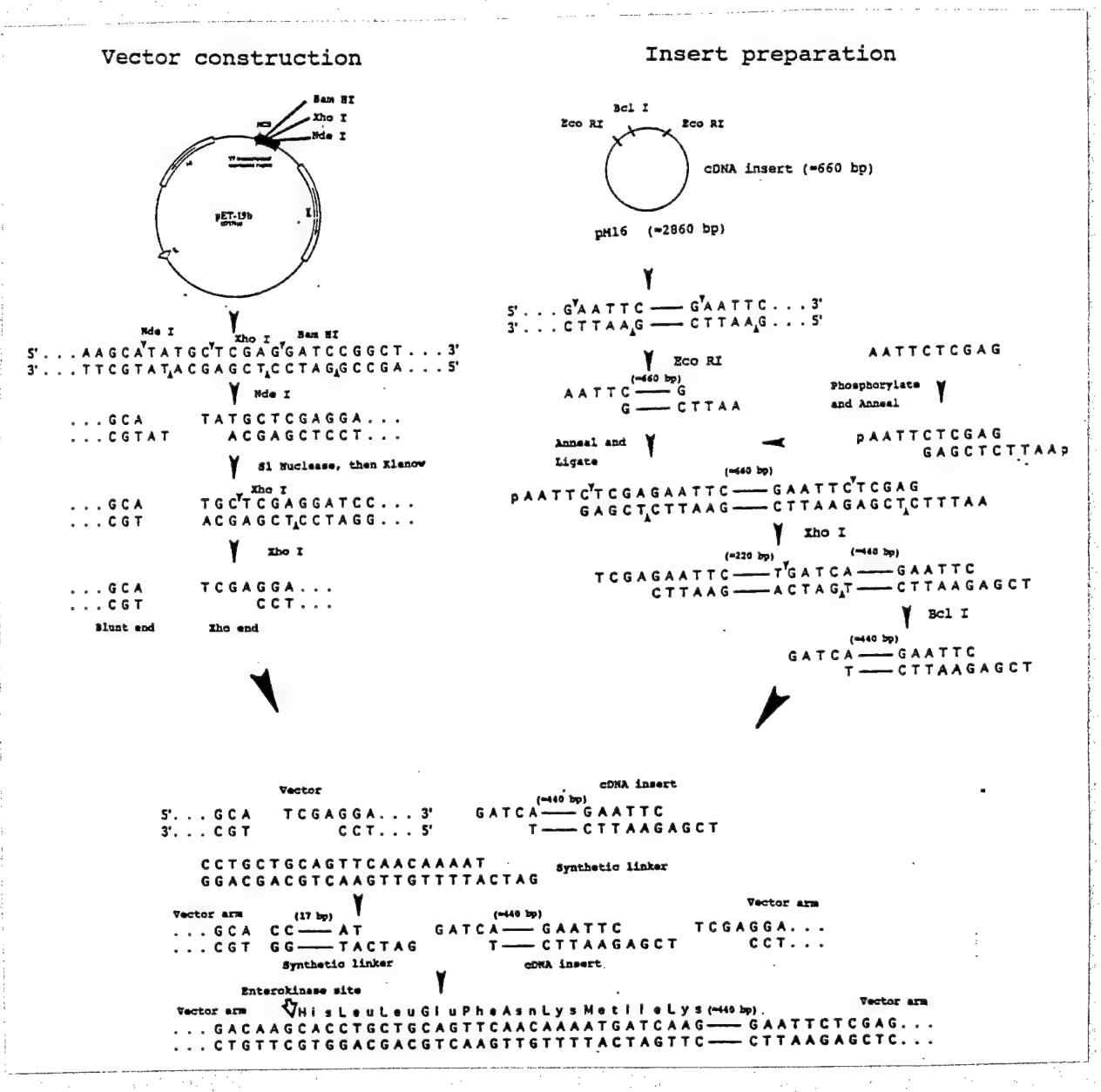


Fig. 12. Construction of pTJ7-133#a16. The insert was prepared as outlined in the above scheme and contained the cDNA fragment containing the coding sequence for the basic subunit of Mojave toxin. It was cloned into expression vector pET-19b as shown. A synthetic linker and adaptor were synthesized to generate the final construct, which included an enterokinase site immediately adjacent to the N-terminal His of the basic subunit protein.

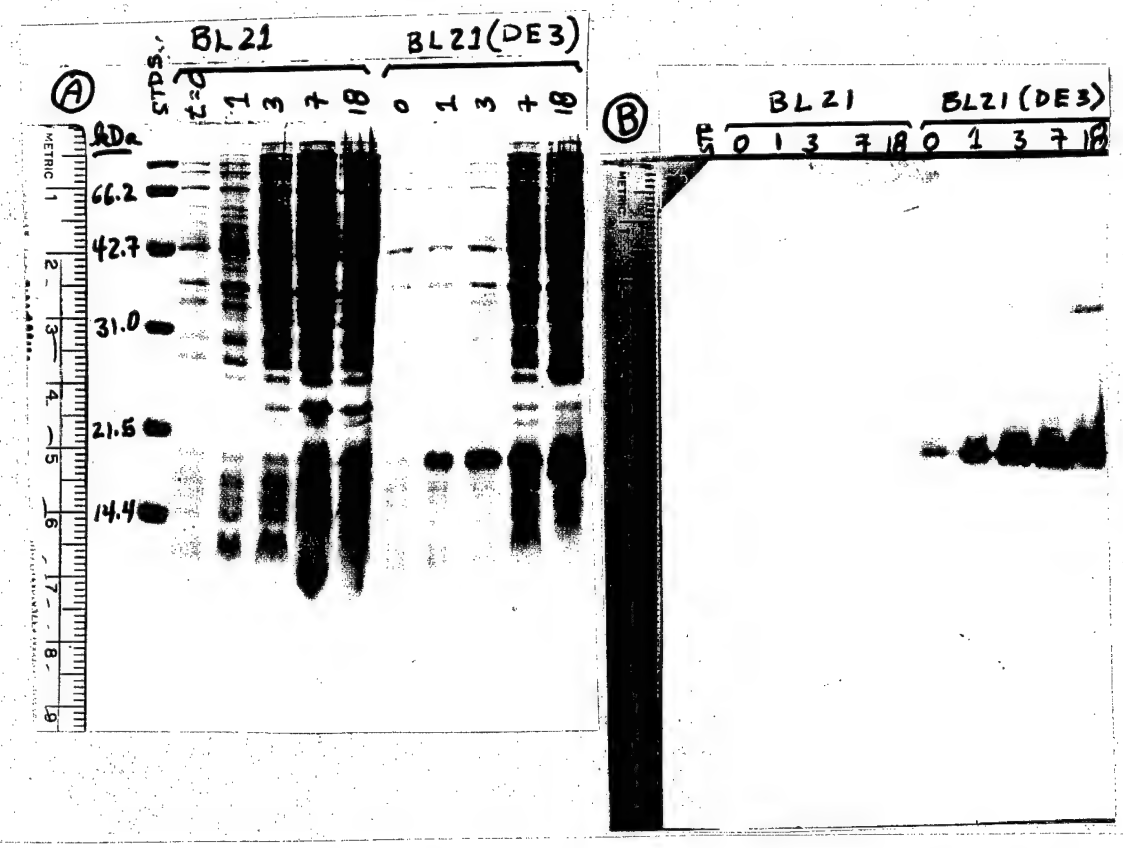


Fig. 13. (A) Coomassie stained proteins following SDS-PAGE on a 15% gel of *E. coli* BL21 and BL21(DE3) samples containing pTJ7-133#a16 (basic subunit of Mojave toxin) after induction with 1mM IPTG. Cells were removed at 0, 1, 3, 7 and 18 hrs after addition of IPTG, centrifuged, resuspended in protein solubilizing solution and applied to the gel. (B) Western blot of a gel identical to (A). Primary antibody used was raised in rabbits against the basic subunit of Mojave toxin. Amounts of protein added per slot were not constant in this experiment.

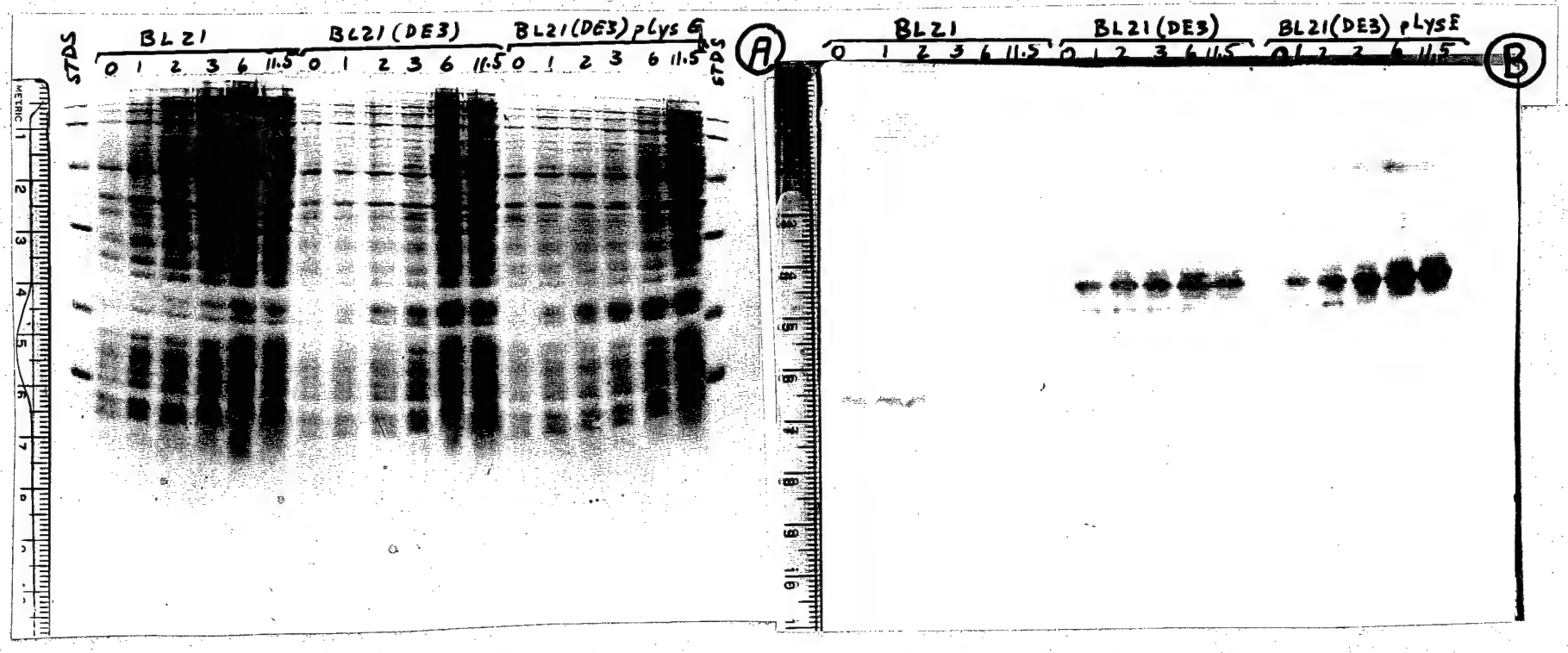
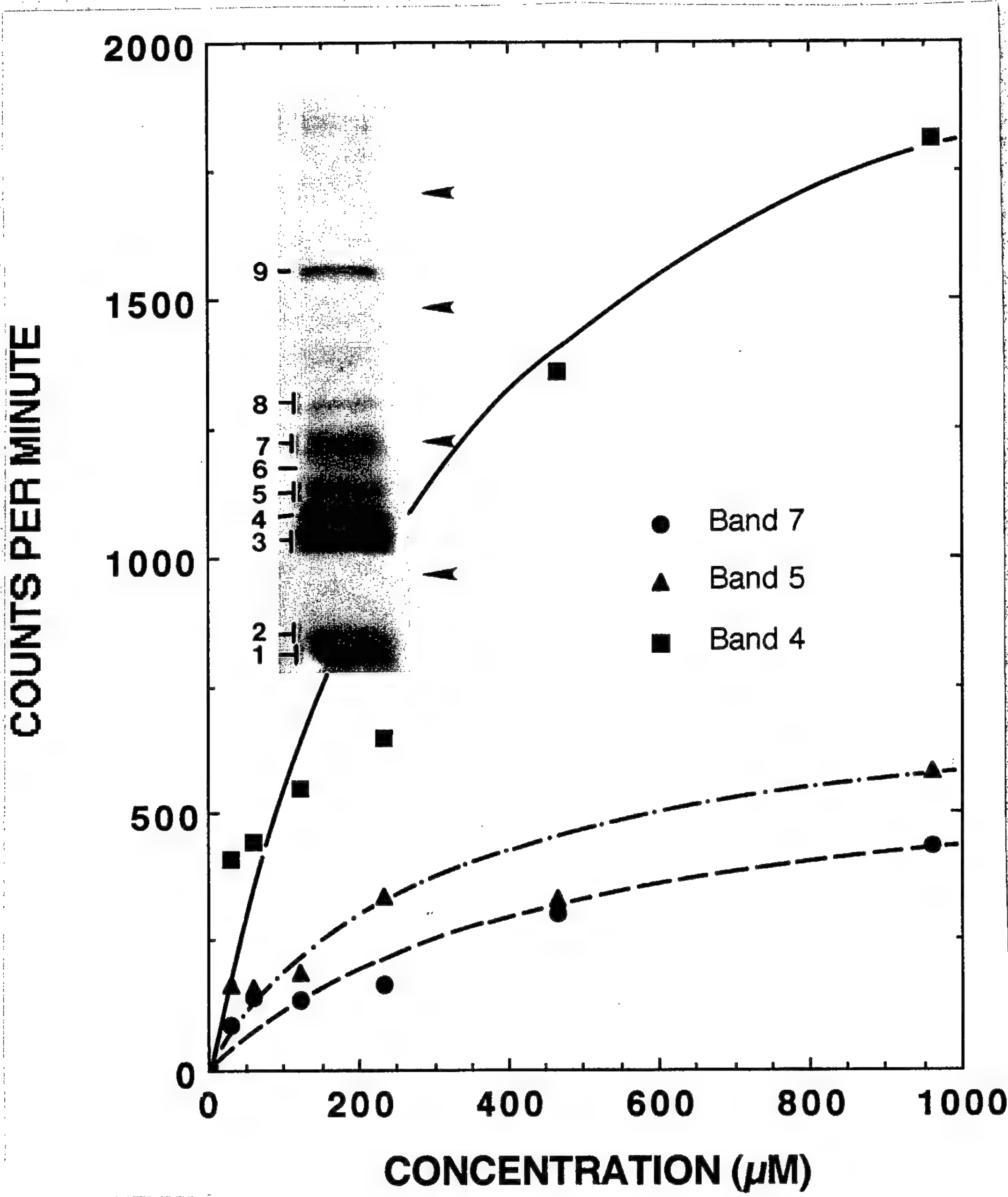


Fig. 14. (A) Coomassie stained proteins following SDS-PAGE on a 15% gel of *E. coli* BL21, BL21(DE3), and BL21(DE3)pLysE samples containing pTJ8-38#3 plasmid (acidic subunit of Mojave toxin) after induction with 1mM IPTG. Cells were removed at 0, 1, 2, 3, 6, and 11.5 hrs after addition of IPTG, centrifuged, resuspended in protein solubilizing solution and applied to the gel. (B) Western blot of a gel identical to (A). Primary antibody used was raised in rabbits against the acidic subunit of Mojave toxin and shown in other experiments not to cross-react with the basic subunit. Amounts of protein added per slot were not constant in this experiment.



MOJAVE TOXIN BASIC SUBUNIT

	1				5					10					15					20					25					30					35					40		
Basic subunit Peptide	H	L	L	Q	F	N	K	M	I	K	F	E	T	R	K	N	A	I	P	F	Y	A	F	Y	G	C	Y	C	G	W	G	G	R	G	R	P	K	D	A	T		
					45					50					55					60					65					70					75					80		
Basic subunit Peptide	D	R	C	C	F	V	H	D	C	C	Y	G	K	L	A	K	C	N	T	K	W	D	I	Y	P	Y	S	L	K	S	G	Y	I	T	C	G	K	G	T	W		
					85					90					95					100					105					110					115					120		
Basic subunit Peptide	C	E	E	Q	I	C	E	C	D	R	V	A	A	E	C	L	R	R	S	L	S	T	Y	K	Y	G	Y	M	F	Y	P	D	S	R	C	R	G	P	S	E	T	C

Fig. 15. Amino acid sequence of the basic subunit of Mojave toxin as determined by Arid, Kruggel, and Kaiser (1990a).

Fig. 16. Incorporation of ^{32}P into three different electrophoretic bands of *N. s. scutatus* venom with increasing concentrations of [$\gamma^{32}P$]-8-N_3ATP. Insert illustrates a typical separation of *N. s. scutatus* venom proteins achieved on 18% acrylamide gels following staining with Coomassie blue. Arrows illustrate mobility of mol. wt. markers with kDa-values of 14.4, 21.5, 31 and 45. Autoradiography of the intact gels showed distinct radioactive bands (data not shown). Bands 4, 5, and 7 were cut and their radioactivity values determined by scintillation counting. Reaction mixtures at the indicated concentration of [$\gamma^{32}P$]-8-N_3ATP were prepared as described in the footnote to Table 3. A control sample electrophoresed without photolysis showed no radioactivity present, indicating no protein kinases were active under our incubation conditions.

Table 1. Expression clones prepared for the basic and acidic subunits of Mojave toxin, and their expression vectors are listed in the upper part of Table 1. The lower half of the table lists the site-specific mutants of the basic subunit of Mojave toxin that have been constructed. Amino acid substitutions are listed for each mutant.

MOJAVE TOXIN EXPRESSION CONSTRUCTIONS

plasmid name	vector	notebook ref/cell stg	mtx subunit/leader
pIK 32-146 #2	pTM-N	IK 32-146 to 148/ Box 4, pos 19b	basic/ompA
pTJ 7-133 #a16	pET 19b	TJ 7-144/ Box 9, pos 5	basic/poly His
pTJ 8-38 #3	pET 19b	TJ 8-45/ Box 9, pos 12	proacidic/poly His
pTJ 8-142 #1	pET 17xb	TJ 8-151/ Box 9, pos 20	proacidic/T7 *gene 10*
pTJ 8-142 #27	pET 17xb	TJ 8-151/ Box 10, pos 2	basic/T7 *gene 10*
pTJ 10-79 #1	pET 17xb*	TJ 10-85/ Box 11, pos 7	proacidic/"met-mtx-a"
pTJ 10-79 #17	pET 17xb*	TJ 10-85/ Box 11, pos 8	basic/"met-mtx-b"
pTJ 10-79 #28	pET 17xb*	TJ 10-85/ Box 11, pos 9	basic/"met-ser-mtx-b"

* For these constructs, the 260 aa *gene 10* leader has been removed and replaced with a met (transcription start) residue. For #28, the his residue normally found in pos 1 has been replaced with a ser residue.

MTX SITE-DIRECTED MUTANTS

plasmid name	vector	notebook ref/cell stg	mtx subunit/identity
pTJ 9-93 #1	pET 17xb	TJ 10-7/ Box 10, pos 19	basic/ K38Q of pTJ 8-142 #27
pTJ 11-28 #1	pET 17xb*	TJ 11-35/ Box 12, pos 4	basic/ K38Q of pTJ 10-79 #17
pTJ 11-28 #7	pET 17xb*	TJ 11-35/ Box 12, pos 5	basic/ Y115D of pTJ 10-79 #17
pTJ 11-28 #13	pET 17xb*	TJ 11-35/ Box 12, pos 6	basic/ K38Q of pTJ 10-79 #28
pTJ 11-28 #19	pET 17xb*	TJ 11-35/ Box 12, pos 7	basic/ Y115D of pTJ 10-79 #28

Table 2. Toxicity assays on mice, using i.v. injections of notechis III-4 and various snake plasma fractions.

Sex	Concentration notechis III-4[a]	% Plasma[b]	Plasma source[c]	Deaths/# mice injected	
F	0.05 μg/g	0	-	0/7	
F	0.10 μg/g	0	-	5/7	
F	0.15 μg/g	0	-	8/8	
F	0.15 μg/g	10	N.s.s.	1/1	
F	0.15 μg/g	20	N.s.s.	1/1	
F	0.15 μg/g	0	N.s.s.	2/3	
F	0.15 μg/g	40	N.s.s.	3/3	
F	0.15 μg/g	60	N.s.s.	2/3	
F	0	20	N.s.s.	0.3	
F	0.15 μg/g	0	-	2/3	
F	0.15 μg/g	20	P.t.	3/3	
F	0.15 μg/g	20	A.a.	3/3	
F	0.15 μg/g	20	O.m.	0/3	Flyer?
M	0.15 μg/g	0	-	3/3	
M	0.15 μg/g	40	P.t.	3/3	
M	0.15 μg/g	40	A.a.	3/3	
M	0.15 μg/g	40	O.m.	3/3	
M	0	20	P.t.	0/3	Plasma
M	0	20	A.a.	0/3	not
M	0	20	O.m.	0/3	toxic

[a]Notechis III-4 is a postsynaptic neurotoxin isolated from the venom of N.s.scutatus (see Francis et al., J. Nat. Toxins 2, 85-101, 1993). Individual mice were weighed and injected i.v. in either of the dorsolateral caudal tail veins as previously described (Aird and Kaiser, Toxicon 23, 361-374, 1985).

[b]Toxin was dissolved in PBS-0.1% BSA. When plasma was added it was substituted for the equivalent volume of PBS-0.1% BSA, so that a 10% plasma sample contained 0.9 ml PBS-0.1% BSA and 0.1 ml plasma.

[c]Sera sources: N.s.s., *Notechis scutatus* (Lake Albert, S.A.); P.t., *Pseudonaja textilis* (S.A.); A.a., *Acanthophis antarcticus* (Gold Coast, QLD); O.m., *Oxyuranus microlepidotus* (Goyders Lagoon, S.A.). Whole blood was collected by cardiac puncture from live, restrained, un-anaesthetized snakes; centrifuged for 20 min, and the sera snap frozen and freeze dried. It was reconstituted with dH_2O immediately before use.

Table 3. Photoincorporation of [γ-^{32}P]-8-N_3ATP into band 4 in the absence and presence of unlabeled nucleotides*

Expt. 1	Competing Nucleotide ATP (mM)	CPM (% incorporated)
	0	15,100 (100)
	1.2	12,200 (81)
	2.4	11,000 (73)
	4.8	10,400 (69)

Expt. 2	CPM (% incorporated)				
		Competing Nucleotide			
Conc. (mM)		ATP	ADP	CTP	CDP
0	12,000 (100)	-	-	-	-
1.2	-	8,300 (69)	10,900 (91)	9,700 (81)	-
Expt. 3					
0	22,900 (100)	-	-	-	-
1.2	-	14,200 (62)	16,900 (74)	-	19,300 (84)

*Crude *N. s. scutatus* venom (Venom Supplies, Tanunda, South Australia) was dialyzed exhaustively against water at 4°C to remove free nucleotides and lyophylized. Dried venom was weighed and dissolved in 20mM Tris-HCl-2.5mM $MgCl_2$ (pH 7.5). Unlabeled nucleotides were weighed and stock solutions prepared in the above Tris-$MgCl_2$ buffer. Stock solutions of known concentrations of radioactive [γ^{32}P]-8-N_3ATP and unlabeled 8-N_3ATP in methanol were purchased from RPI (Mt. Prospect, IL, U.S.A.). Specific activities of labeled [γ^{32}P]-8-N_3ATP were adjusted with unlabeled 8-N_3ATP to 240-446 mCi/mmole in different experiments. Photoaffinity labeling followed the general procedures outlined by Potter and haley (1983) as well as the "Photoaffinity Labeling" manual provided by RPI. Each reaction mixture (50μl) contained a final concentration of 20mM Tris-HCl-2.5mM $MgCl_2$ (pH 7.5); 400μM [γ^{32}P]-8-N_3ATP; and 20-30μg dialyzed crude protein. This solution was incubated 1 min on ice and photolyzed for 2 min on ice at 254nm with a UVA-11 mineral lamp (Potter and Haley, 1983). Forty μl of solubilizing solution containing 70mM Tris-HCl (pH 6.8), 43% sucrose, 0.029% bromophenol blue, 100mM SDS, 2.5mM EDTA, and 200mM DTT was added to each reaction mixture, it was stirred, incubated at room temperature for 2 min, and kept on ice until all samples were prepared. Samples were then heated to 90°C for 1 min, microfuged, and 60μl transferred to a well on a 18% acrylamide slab gel (1.5mm thick) (Jorge da Silva *et al.*, 1989). Following electrophoresis, gels were stained with Coomassie blue, destained with 10% isopropanol-5% acetic acid for 1 hr, and washed 3X with water. The hydrated gel was covered with Saran wrap, the wrap was marked with radioactive ink, and exposed to Kodak X-O Mat-AR film using an intensifier. Bands from gels were either cut directly from the wet gel or first precipitated by soaking the gels in 93% ethanol-7% glycerol solution and then cut. In experiments where nucleotides were used to compete with [γ^{32}P]-8-N_3ATP binding, the proteins were first mixed with the unlabeled nucleotides at the indicated concentration for 1 to 3 min on ice, [γ^{32}P]-8-N_3ATP added, and incubated on ice for another min before the two min photolysis, and then treated as previously described. (-) indicates not determined.

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